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Energetic Limitations Of Thermophilic Methanogens And Thiosulfate  
Reducers In The Subsurface Biosphere At Deep-Sea Hydrothermal Vents

A Dissertation Presented

By

LUCY C. STEWART

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfilment  
of the requirements for the degree of

Doctor of Philosophy

September 2015

Microbiology Department



Energetic Limitations Of Thermophilic Methanogens And Thiosulfate Reducers In The  
Thermophilic Subsurface Biosphere At Deep-Sea Hydrothermal Vents

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LUCY C. STEWART

Approved as to style and content by:

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Microbiology

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Our research relies heavily on collaborators and support to create a clear picture of activity at deep-sea hydrothermal vents – not the easiest of environments to sample. I would like to thank our fieldwork collaborators for this research – Julie Huber, Caroline Fortunato, Joe Vallino, Chris Algar, Dave Butterfield, Ben Larson and Giora Proskurowski. I would also like to acknowledge the crews of the R/V *Falkor*, the R/V *Marcus G. Langseth*, and the R/V *Ronald H. Brown*, as well as the operating teams of ROV *Jason II* and ROV *ROPOS*.

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## ABSTRACT

# ENERGETIC LIMITATIONS OF THERMOPHILIC METHANOGENS AND THIOSULFATE REDUCERS IN THE THERMOPHILIC SUBSURFACE BIOSPHERE AT DEEP-SEA HYDROTHERMAL VENTS

SEPTEMBER 2015

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This dissertation examined the substrate and energetic limitations of hydrogenotrophic thermophiles from deep-sea hydrothermal vents. Thermophilic and hyperthermophilic organisms in diffuse hydrothermal venting are thought to represent a hot subsurface biosphere associated with deep-sea hydrothermal vents, where primary production is dominated by hydrogenotrophy rather than sulfide oxidation as at the vent/seawater interface of hydrothermal sulfide chimneys. Methanogens and sulfur-reducers are known to compete for hydrogen in mesophilic, freshwater systems, and likely do so in deep-sea hydrothermal vent environments as well. However, the exact size and biomass of the subsurface biosphere is difficult to determine through direct sampling.

Firstly, the distribution of thermophilic and hyperthermophilic methanogens, sulfur-reducers, and heterotrophs in diffuse venting fluids at our field site, Axial Volcano (on the Juan de Fuca Ridge), was examined using culture-dependent (Most-Probable-Number) and independent (omics) techniques. It was confirmed that methane production in diffuse venting fluids could be stimulated by the sole addition of hydrogen and incubation at thermophilic and/or hyperthermophilic temperatures, indicating that methanogens in this system are not limited significantly by nutrient or trace element

requirements. To determine why one novel hyperthermophilic methanogen from our field site (*Methanocaldococcus bathoardescens*) appeared to prefer high levels of nitrogen when grown in the lab, its genome was examined for nitrogen assimilation-related genes.

In the laboratory, the growth energies of *Methanocaldococcus* and *Methanothermococcus* spp. over their full temperature ranges were measured in order to determine Arrhenius constants for their production of methane. They were also grown in continuous flow chemostat culture to determine their hydrogen limitations at both optimal and sub-optimal temperatures for growth, and the Monod kinetics for their hydrogen use and methane production were measured. Additionally, the minimum hydrogen and thiosulfate requirements, as well as Monod kinetics, were measured in batch bioreactor culture for a thiosulfate-reducing, hydrogenotrophic, thermophilic *Desulfurobacterium* sp. isolated from another site on the Juan de Fuca Ridge, the Endeavour Segment, to determine where it might compete with methanogens for hydrogen.

Finally, the geochemical and distribution data from Axial Volcano and laboratory-derived kinetic data for thermophilic and hyperthermophilic methanogens were used to create a one-dimensional reactive transport model (RTM) of hydrogenotrophic methanogenesis in the subsurface at Axial Volcano. In this way, the relative dimensions and biomass of methanogens in the subsurface can be predicted without direct sampling. In future, this type of model could be used make predictions about the thermophilic subsurface at other vent locations, as well as expanded to include competition between different types of hydrogenotrophs (rather than just hydrogenotrophic methanogens with different optimum temperatures) and interactions with other organisms, such as hydrogen-producing hyperthermophilic heterotrophs.

# TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS .....	iv
LIST OF TABLES .....	xi
LIST OF FIGURES .....	xii
1 INTRODUCTION .....	1
1.1 Objectives and Hypotheses .....	1
1.2 Astrobiology.....	3
1.2.1 Definitions of Astrobiology .....	3
1.2.2 Defining Habitability .....	4
1.2.3 Habitability in Extraterrestrial Environments .....	6
1.3 Deep-Sea Hydrothermal Vent Systems and the Subseafloor Biosphere.....	7
1.3.1 Hydrothermal Vent Systems as Extraterrestrial Analogs .....	7
1.3.2 Axial Volcano .....	13
1.3.3 High Rise Field, Endeavour Segment.....	16
1.4 Hyperthermophilic Anaerobes in Hydrothermal Vent Systems and the Subsurface Biosphere .....	18
1.4.1 Molecular Surveys of Hyperthermophile Diversity.....	18
1.4.2 Thermophilic and Hyperthermophilic Methanogens .....	20
1.4.3 Thermophilic Sulfur Reducers .....	24



1.4.4	Other Hyperthermophilic Organisms at Hydrothermal Vents .....	27
1.5	Modelling of Hydrothermal Vent Systems .....	28
1.5.1	Prior Geochemical Modelling of Habitability at Vent Systems .....	28
1.5.2	Microbial Maintenance Energies .....	30
1.5.3	Reactive Transport Flow Modelling and Modelling Parameters .....	32
1.6	Summary and Research Approach .....	36
2	REACTIVE TRANSPORT MODEL OF METHANOGENESIS IN THE SUBSEAFLOOR OF THE AXIAL VOLCANO HYDROTHERMAL FIELD .....	38
2.1	Abstract .....	38
2.2	Introduction .....	39
2.3	Methods .....	42
2.3.1	Chemostat data collection .....	42
2.3.2	Batch experiments .....	44
2.3.3	Most-Probable-Number (MPN) and microcosm experiments .....	45
2.3.4	Metagenome and metatranscriptome analysis .....	47
2.4	Results and Discussion .....	50
2.4.1	Geochemistry and microbiology at Axial Volcano .....	50
2.4.2	Methane production experiments .....	58
2.4.3	Reactive transport modelling in the hydrothermal vent subsurface .....	64

3	THERMOPHILIC, CHEMOLITHOAUTOTROPHIC SULFUR-REDUCING <i>DESULFUROBACTERIUM</i> SP. KINETICALLY OUTCOMPETES METHANOGENS FROM DEEP-SEA HYDROTHERMAL VENTS FOR HYDROGEN.....	69
3.1	Abstract .....	69
3.2	Introduction .....	70
3.3	Materials and Methods .....	73
3.3.1	Field sampling and redox energy estimates .....	73
3.3.2	Isolation of new thermophile strains.....	74
3.3.3	Cell characteristics .....	75
3.3.4	Phylogenetic analyses .....	76
3.3.5	Electron microscopy .....	77
3.3.6	Growth conditions.....	77
3.4	Results .....	79
3.4.1	Fluid chemistry and reaction energetics .....	79
3.4.2	Characteristics of strains HR11 and BW11 .....	81
3.4.3	Monod kinetics for <i>Desulfurobacterium</i> strain HR11 .....	84
3.5	Discussion .....	85
4	TAXONOMY AND GENOME OF <i>METHANOCALDOCOCCLUS</i> <i>BATHOARDESCENS</i> SP. NOV.....	92
4.1	Abstract .....	92

4.2	Introduction .....	93
4.3	Materials and Methods .....	94
4.3.1	Genome sequencing .....	94
4.3.2	Phylogenetic analysis .....	94
4.3.3	Genome analysis .....	95
4.4	Results and Discussion .....	95
4.4.1	Phylogenetic tree .....	95
4.4.2	Genome similarity indices .....	97
4.4.3	Notable genome features .....	99
4.4.4	Description of <i>Methanocaldococcus bathoardescens</i> , sp. nov. ....	100
5	SUMMARY AND CONCLUSIONS .....	102
	BIBLIOGRAPHY .....	107

## LIST OF TABLES

Table	Page
2.1: Axial Volcano diffuse hydrothermal fluid characteristics .....	51
2.2: Most-Probable-Number (MPN) estimates at 80°C for the 2012-2013 Axial Volcano cruises. Cell concentrations are in cells L <sup>-1</sup> , with the three-tube MPN scores in brackets (*microcosm run, **microcosm growth).....	55
2.3: Most-Probable-Number (MPN) estimates at 55°C for the 2012-2013 Axial Volcano cruises. Cell concentrations are in cells L <sup>-1</sup> , with the three-tube MPN scores in brackets (*microcosm run, **microcosm growth).....	56
2.4: End-point methane concentrations (total µmoles in headspace) following incubation of hydrothermal fluid with varying amounts of H <sub>2</sub> in the headspace with and without the addition of 47 µM NH <sub>4</sub> Cl .....	57
2.5: Monod constants ( $k_s$ and $V_{max}$ ) for <i>M. jannaschii</i> , <i>M. thermolithotrophicus</i> , and <i>Methanothermococcus</i> sp. BW11. ....	63
3.1: Chemical composition of end-member hydrothermal vent fluid from the Boardwalk edifice extrapolated to zero-Mg <sup>2+</sup> from this study and seawater for modeling purposes “Seawater composition from Amend <i>et al.</i> (2011), except the O <sub>2</sub> concentration which is from Von Damm <i>et al.</i> (1985). ....	80
4.1: Differential characteristics of <i>Methanocaldococcus</i> species .....	97
4.2: ANI and GGDC analyses of genomic DNA from strain JH146 <sup>T</sup> and related species of the genus <i>Methanocaldococcus</i> . Data in bold type represent the closest relatives. DDH, DNA-DNA homology. GenBank/EMBL/DDBJ genome accession numbers: strain JH146 <sup>T</sup> , CP009149.1; strain FS406-22, CP001901.1; <i>M. jannaschii</i> DSM2661 <sup>T</sup> , L77117.1 (Bult <i>et al.</i> , 1996); <i>M. fervens</i> AG86 <sup>T</sup> , CP001696.1; <i>M. infernus</i> ME <sup>T</sup> , CP002009.1; <i>M. vulcanius</i> M7 <sup>T</sup> , CP001787.1.....	98
4.3: Genome statistics .....	100

## LIST OF FIGURES

Figure	Page
1.1: Global distribution of hydrothermal venting. S. Beaulieu, K. Joyce, and S.A. Soule (WHOI), 2010, <a href="http://www.interridge.org/irvents/maps">http://www.interridge.org/irvents/maps</a> . Funding from InterRidge and Morss Colloquium Program at WHOI. ....	9
1.2: Volcanism and hydrothermal venting on the seafloor(Schrenk <i>et al.</i> , 2010). ....	10
1.3: Diagram illustrating the alteration of seawater to hydrothermal fluid via water/rock reactions in the heated crustal zone near upwelling magma. The hot, reducing, metal-rich hydrothermal fluid can either return to the ocean floor directly and precipitate out metal-sulfide ‘chimneys’ as it emerges into the cold, oxic ocean, or it can be progressively diluted by unaltered seawater and emerge as low-temperature, ‘diffuse’ hydrothermal fluid. Subsurface hydrothermal fluid/seawater mixtures below approximately 120°C host the subsurface biosphere. ....	11
1.4: Location of Axial Volcano and the Juan de Fuca Ridge (www.pmel.noaa.gov/eoi/nemo).....	14
1.5: Phylogenetic tree of the methanogens, also showing relationship to the Thermococcales. Created using the Ribosomal Database Project Tree Builder ( <a href="http://rdp.cme.msu.edu/treebuilder/treeing.spr">http://rdp.cme.msu.edu/treebuilder/treeing.spr</a> ). Hyperthermophilic groups are in red. Bar, 3% sequence divergence. ....	23
1.6: Phylogenetic tree of the phylum Aquificae. Created using the Ribosomal Database Project Tree Builder ( <a href="http://rdp.cme.msu.edu/treebuilder/treeing.spr">http://rdp.cme.msu.edu/treebuilder/treeing.spr</a> ). Groups associated with deep-sea hydrothermal vent systems, including the <i>Desulfurobacteria</i> , are in blue. Bar, 2% sequence divergence. ....	26
1.7: 1-D model of hydrothermal fluid and seawater mixing to produce diffuse venting fluid. ....	34
2.1: Site map of Axial Volcano showing hydrothermal vent locations and sampling sites. The green circles show the locations of background seawater hydrocasts. The inset shows the location of Axial Volcano in the northeastern Pacific Ocean. ....	50
2.2: Metagenomics and metatranscriptomics of selected vents at Axial Volcano.....	53
2.3: Microcosm and chemostat experiments. A and B: methane production by microcosm cultures from Axial Volcano diffuse hydrothermal venting at 55°C (A) and 80°C (B); high H <sub>2</sub> is red, high H <sub>2</sub> + NH <sub>4</sub> Cl is blue, low H <sub>2</sub> is green.. C: methane production per cell in continuous flow chemostat culture by <i>Methanocaldococcus jannaschii</i> (82°C, red and 65°C, black). D: methane production per cell in continuous flow chemostat culture by <i>Methanothermococcus thermolithotrophicus</i> (65°C, red and 55°C, black).....	59

2.4: Arrhenius kinetics of thermophilic and hyperthermophilic methanogens grown in Balch tubes. <i>M. jannaschii</i> : $A = 4.41 \times 10^{-4}$ mol CH <sub>4</sub> /cell/h, $E_a = 64.33$ kJ/mol. <i>M. thermolithotrophicus</i> : $A = 4.91 \times 10^{-4}$ mol CH <sub>4</sub> /cell/h, $E_a = 61.36$ kJ/mol. ....	60
2.5: Growth energies of <i>M. jannaschii</i> , <i>M. bathoardescens</i> , and <i>M. thermolithotrophicus</i> at different concentrations of NH <sub>4</sub> Cl. <i>M. bathoardescens</i> data are from Ver Eecke <i>et al.</i> (2013). ....	61
2.6: Temperature of mixed fluid vs. proportion of seawater to hydrothermal fluid. Growth of methanogens is only possible where the temperature is below 120°C. ....	65
3.1: Boardwalk hydrothermal vent sampling site showing the black smoker (bottom) that was the source of the 341°C hydrothermal fluid and the tubeworm mound (left side) that was the source of the 19°C fluid. The image is a video frame grab from ROV <i>Jason II</i> dive J2-576 at 18:46:15 on 07/22/2011. ....	72
3.2: Predicted catabolic energies (per kg of mixed fluid) available for hydrogenotrophic sulfate reduction (●), hydrogenotrophic methanogenesis (○), aerobic sulfide oxidation (▲), and aerobic methane oxidation (Δ) at varying temperatures in mixed abiotic hydrothermal-seawater solutions flowing from the Boardwalk edifice. ....	81
3.3: Neighbor-joining trees showing the positions of (A) strain HR11 within the genus <i>Desulfurobacterium</i> (870 nt) and (B) strain BW11 within the genus <i>Methanothermococcus</i> (880 nt) based on sequences of the 16S rRNA gene. GenBank/EMBL/DDBJ accession numbers are included in parentheses. The topology of the tree was estimated by bootstraps based on 500 replications. Numbers at the branch point are the percentage support by bootstraps. Bar, 2% sequence divergence. ....	83
3.4: Growth rates for strain HR11 grown over its ranges of temperature (A), pH (B), and NaCl concentration (C). Error bars represent 95% confidence intervals. ....	84
3.5: Growth rates for strain HR11 grown over its ranges of H <sub>2</sub> concentration (A) and initial Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> concentration (B). The line is a Michaelis-Menten fit to the data. ....	85
4.1: Neighbor-joining tree showing the position of strain JH146 <sup>T</sup> within the genus <i>Methanocaldococcus</i> based on sequences of the 16S rRNA gene (1293 nt). GenBank/EMBL/DDBJ accession numbers are included in parentheses. The topology of the tree was estimated by bootstraps based on 500 replications. Numbers at the branch point are the percentage support by bootstraps. Bar, 2% sequence divergence. ....	96

# CHAPTER 1

## INTRODUCTION

### 1.1 Objectives and Hypotheses

Astrobiology as a field encompasses everything from the search for extrasolar planets to charting the history of life on our own. One of its key foci has always been establishing the limits of life “as we know it” – namely life on Earth (Des Marais *et al.*, 2008).

Traditionally, the search for habitable locations has focused on “following the [liquid] water”, given that all life on Earth requires liquid water at some stage and scale. A newer approach, however, has been to “follow the energy”. In this paradigm, habitability is analyzed in terms of the energy available for biological utilization (Hoehler *et al.*, 2007), often using analogue habitats on Earth which harbor extremophilic organisms. One particularly well-studied analog extraterrestrial environment is deep-sea hydrothermal vents. As a habitat, vents are notable for having ecosystems based on chemosynthetic primary production, rather than the photosynthetic primary production that underlies all surface ecosystems. In hydrothermal vent systems, primary producers exploit not the sun’s light, but the chemical disequilibrium between hot, reducing hydrothermal fluid and cold, oxygenated seawater (McCollom and Shock, 1997). Distribution of chemolithoautotrophic organisms at hydrothermal vent systems has been modeled using an energetic approach, calculating energy available for specific metabolic reactions (e.g. methanogenesis) from the geochemical composition of hydrothermal fluids and seawater, and comparing these to rates of energy usage by vent organisms (McCollom and Shock, 1997; McCollom, 2007). However, these energy usage rates are often based on

theoretical minima such as the energy required to produce one molecule of ATP (Hoehler, 2004). Accurate measurements of energy usage by appropriate model organisms are limited. Whether measured as “growth energies”, minimum energy requirements for reproduction, or true “maintenance energies”, for metabolic turnover without growth (Morita, 1999), they have largely been gathered for aerobic, mesophilic heterotrophs (Tijhuis *et al.*, 1993). There are few energetic requirement measurements for anaerobes at thermophilic and hyperthermophilic temperatures – organisms which represent a significant fraction of autotrophic primary production in hydrothermal vent systems. Furthermore, while temperature is thought to be a primary control on the variation of energetic requirements between organisms, it is unknown to what extent this varies over temperature *within* an individual organism’s growth range, or with the availability of substrates for autotrophy. This dissertation aims to examine the energetic requirements of thermophilic and hyperthermophilic chemolithoautotrophs sampled from low-temperature hydrothermal venting, and use it to predictively model the potential contribution of these organisms to the subsurface biosphere.

This dissertation is divided into five chapters. **The first chapter** summarizes current definitions of habitability in astrobiology and previous attempts to model it using energetic characteristics, as well as describing the biology, geology, and geochemistry of our field sites. The **second chapter** describes the energetics and hydrogen requirements of thermophilic and hyperthermophilic methanogenic archaea from hydrothermal vents and how they can be used to create a reactive transport model for methanogenesis, as well as constraining this model through the collection of contemporaneous geochemical and



microbial abundance data from our field site. The **third chapter** describes the growth kinetics and substrate limitations of a thermophilic, chemolithoautotrophic thiosulfate-reducing bacterium, also from a hydrothermal vent, and compares them with comparable data for methanogens to predict the outcome of competition between the two functional groups. **The fourth chapter** investigates how the genome of a previously-characterized *Methanocaldococcus* species (*Methanocaldococcus bathoardescens*) may help explain our findings about its growth requirements. Ultimately, this body of work provides a framework for constructing predictive models using geochemical data obtained from extraterrestrial sites. **The fifth chapter** summarizes the findings of this research.

## 1.2 Astrobiology

### 1.2.1 Definitions of Astrobiology

Astrobiology is defined as the search for life beyond Earth. This raises three important questions: what is “life”, where could it be, and how do we look for it? While the first question is primarily the domain of philosophers and biochemists, the second two questions can be addressed by trying to understand the nature of life *on* Earth. What are its limits? Where and how can we find life – of the type we are already familiar with – in extreme environments on our own planet? Can we use those environments, and the organisms that inhabit them, as analogues for extraterrestrial environments (Des Marais *et al.*, 2008; Horneck, 2008)?

In terms of currently searchable extraterrestrial habitats, there are several bodies within our own solar system which probably are or have been capable of supporting life as we know it. These include, primarily, Mars, which appears to have had a warmer and wetter history than its current dry state (McKay, 1986; Ehlmann *et al.*, 2011; Mustard *et al.*,

2008; Wharton *et al.*, 1989), and the Galilean moon of Jupiter, Europa, which conceals a salty ocean beneath its icy crust (Roth *et al.*, 2014). Astrobiologists see ample opportunity for life equivalent to the prokaryotes of Earth – relatively simple, single-celled organisms - to have emerged or been transported to these bodies in the past, and to perhaps still be there. However, these two bodies still form a very large search area, given that life and/or its remnants cannot be ubiquitous on them. Otherwise, we would presumably have detected it already. Some sort of metric is required in order to narrow the search window in a way which enables the detection of life that may not be entirely analogous to the forms we are familiar with, while excluding entirely uninhabitable environments. A traditional strategy has been to “follow the water” – liquid water, at some scale, being a fundamental prerequisite for the growth of all life on Earth (Rothschild and Mancinelli, 2001). This strategy, however, is problematic in that while water is a prerequisite for life on Earth, it is not the *only* prerequisite. A more recent proposal has been that astrobiologists should “follow the energy” (Hoehler, 2007; Hoehler *et al.*, 2007).

### 1.2.2 Defining Habitability

All life – indirectly, through a chain of predation, or directly, by primary production – gains the energy it needs to survive and grow through the exploitation of chemical and energetic disequilibria. Most Earth ecosystems ultimately derive from photosynthetic primary production, which exploits the energy carried by photons, and a small minority from chemosynthetic primary production, which exploits disequilibria between redox pairs of chemicals. In both cases energy is diverted into biological storage, which the cell can draw on for repair and growth, and other organisms can then predate upon. An

environment's "habitability" is a function of its suitability to house specific organisms. While we traditionally think of some environments on Earth as "extreme", this is because they are inhospitable to our own species and its supporting ecosystem. A house is as "extreme" an environment to an anaerobic deep-sea hyperthermophile as a hydrothermal mid-ocean ridge vent is to a human. Shock and Holland (2007) suggest defining habitability via power, that is to say energy demand over time, with the appropriate units being watts per organism. In this approach, an organism's energy requirements in terms of joules per second (i.e. watts) could be compared to the power available from that organism's predominant mode of energy collection. This is a particularly useful approach for chemolithoautotrophs, which gain energy entirely from the exploitation of chemical disequilibria. Moreover, chemolithoautotrophs are the most likely forms of life to either evolve from an abiotic system or persist in an environment where light is not readily available. This is relevant for astrobiology, as the surfaces of both extraterrestrial bodies in the solar system and the early Earth are/were highly inhospitable environments due to ionizing radiation and consistent bombardment by smaller planetary bodies and asteroids.

Hence, one way of approaching the question of habitability is to try and understand how and which chemical disequilibria are available for exploitation by living organisms – how might the hypothetical inhabitants of Europa's ocean or subterranean Mars make their living? This characterization of habitability requires two key parameters: a measurement of energy availability, and an estimate of minimum energy usage requirements. The first can be derived via measurements of the geochemical nature of a potential habitat. The second, absent extraterrestrial organisms to study, can best be estimated through

comparisons to terrestrial organisms inhabiting similar environments. In other words, we must determine the *energetic* boundaries of life on Earth.

### 1.2.3 Habitability in Extraterrestrial Environments

Modelling habitability in extraterrestrial environments is a useful way of establishing parameters for the search for life in them. It is much easier to predict or even directly sample the chemistry of extraterrestrial environments, ancient or modern, than it is to sample their potential biology.

For example, current missions to Mars are examining the geochemistry of ancient Martian environments (Maurice *et al.*, 2012) and monitoring its modern atmosphere (Murchie *et al.*, 2007). The surface of Mars is currently cold, dry, and largely inhospitable to life as we know it, but early Mars could have been a suitable habitat for life (Jakosky and Shock, 1998) and subsurface hydrothermal environments on modern Mars could still be capable of harboring it (Ehlmann *et al.*, 2011; Summers, 2002). Methane has been detected in the Martian atmosphere and appears to be produced in a cyclic fashion (Knak *et al.*, 2014; Webster *et al.*, 2015). Whether it is biotic or abiotic is still unknown.

Another type of potentially habitable environment in the modern solar system is the ice-covered moon – in particular, Europa, the fourth-largest satellite of Jupiter, and Enceladus, the sixth-largest moon of Saturn. Both bodies are entirely surfaced with ice and are thought to harbor subsurface oceans, perhaps even globally, due to gravitational heating (Chyba *et al.*, 2002; McKay *et al.*, 2014). It is plausible that such oceans could have hydrothermal venting which would provide a potential habitat for life (Zolotov and

Shock, 2004) – hydrothermal vents are considered one of the possible locations for the origin of life on Earth (Baross and Hoffman, 1985). While lander missions to investigate the interiors of these bodies have been proposed (Konstantinidis *et al.*, 2015), given that their oceans likely lie below (at minimum) several kilometers of ice, direct sampling poses a great number of difficulties. However, the chemistry of their oceans can be, and has been, indirectly studied by spectroscopic and fly-by probe sampling of plumes offgassing into space (Roth *et al.*, 2014; Hsu *et al.*, 2015; Bouquet *et al.*, 2015). This means that it is possible to model the probable geochemistry of their interiors and the energy available for life (Zolotov and Shock, 2004), but these predictions still require energetic budgets for the organisms themselves (Hoehler, 2013).

In order to establish parameters for the search for life in conditions such as these, it is necessary to understand organisms in similar environments on Earth – such as hydrothermal vent systems.

### **1.3 Deep-Sea Hydrothermal Vent Systems and the Subseafloor Biosphere**

#### **1.3.1 Hydrothermal Vent Systems as Extraterrestrial Analogs**

Deep-sea hydrothermal vents are perhaps most notable in being one of the few habitable environments on Earth that have ecosystems based largely - if not entirely - on chemosynthesis. Predicted to exist at mid-ocean spreading zones prior to their discovery, they were first identified in 1977 (Corliss *et al.*, 1979). They are analogous to terrestrial thermal springs. Both are areas where water drawn is down into the Earth's crust – igneous oceanic crust in the case of deep-sea hydrothermal vents, continental in the case of terrestrial hydrothermal areas - then heated and chemically altered by contact with hot rock, which is close to the crust's surface due to volcanic activity. Venting of the altered

hydrothermal fluid from the crust produces terrestrial thermal springs and deep-sea hydrothermal vents (Corliss *et al.*, 1979; Williams *et al.*, 1979). In the case of deep-sea hydrothermal vents, they are usually associated with mid-ocean ridges. These are the sites of seafloor spreading, circling the globe like the seams on a baseball (Figure 1.1), where new oceanic crust is formed by the upwelling of magma as part of the cycle of plate tectonics (Kelley *et al.*, 2002). On short time-scales, this manifests as volcanic activity. Like areas of volcanic activity in terrestrial environments, volcanic activity in the deep-sea has associated hydrothermal venting. Apart from mid-ocean ridges, deep-sea hydrothermal vents also occur in association with back-arc volcanism – volcanoes formed by the upwelling of magma behind zones of crustal subduction, as the subducted crust melts (Embley *et al.*, 2012) – or hot-spot volcanism, where magma upwells in the middle of a plate, the most notable example of this probably being the Hawai’ian volcanoes (Rubin *et al.*, 2011). Figure 1.2 illustrates the different kinds of volcanism and hydrothermal venting on the seafloor.

## Global Distribution of Hydrothermal Vent Fields

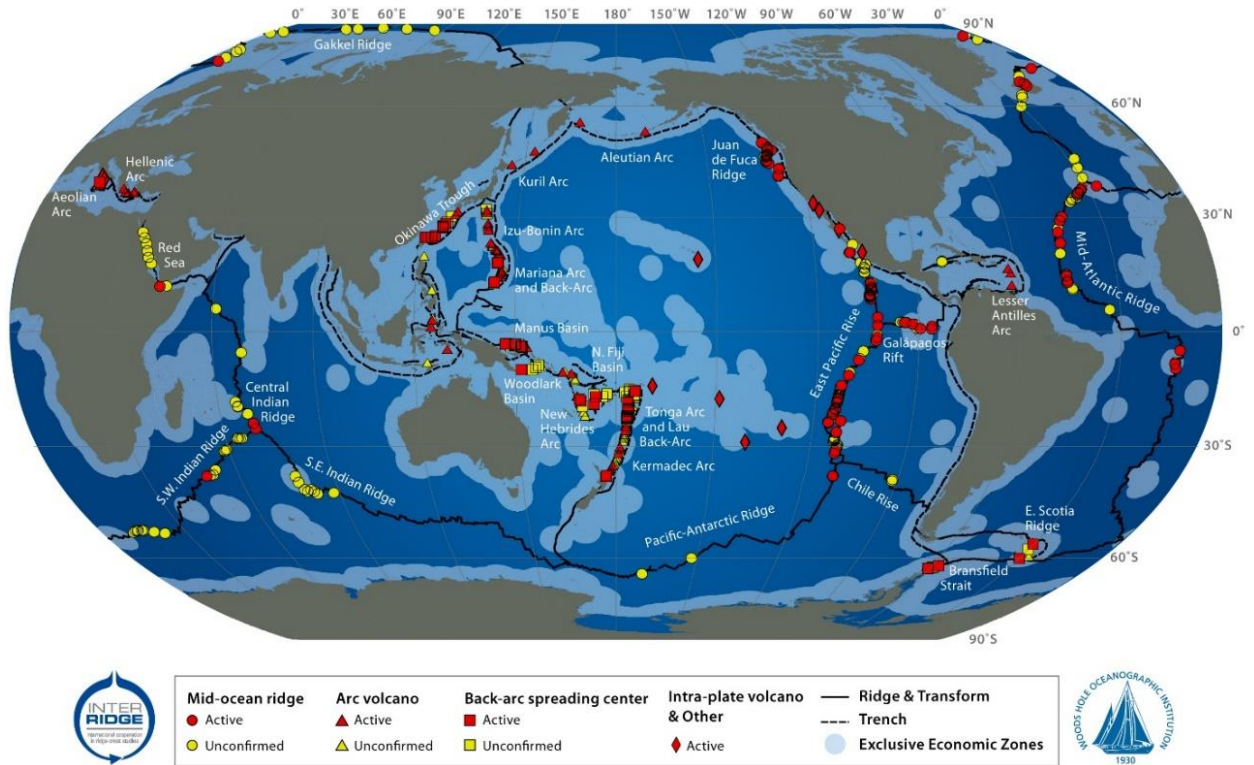
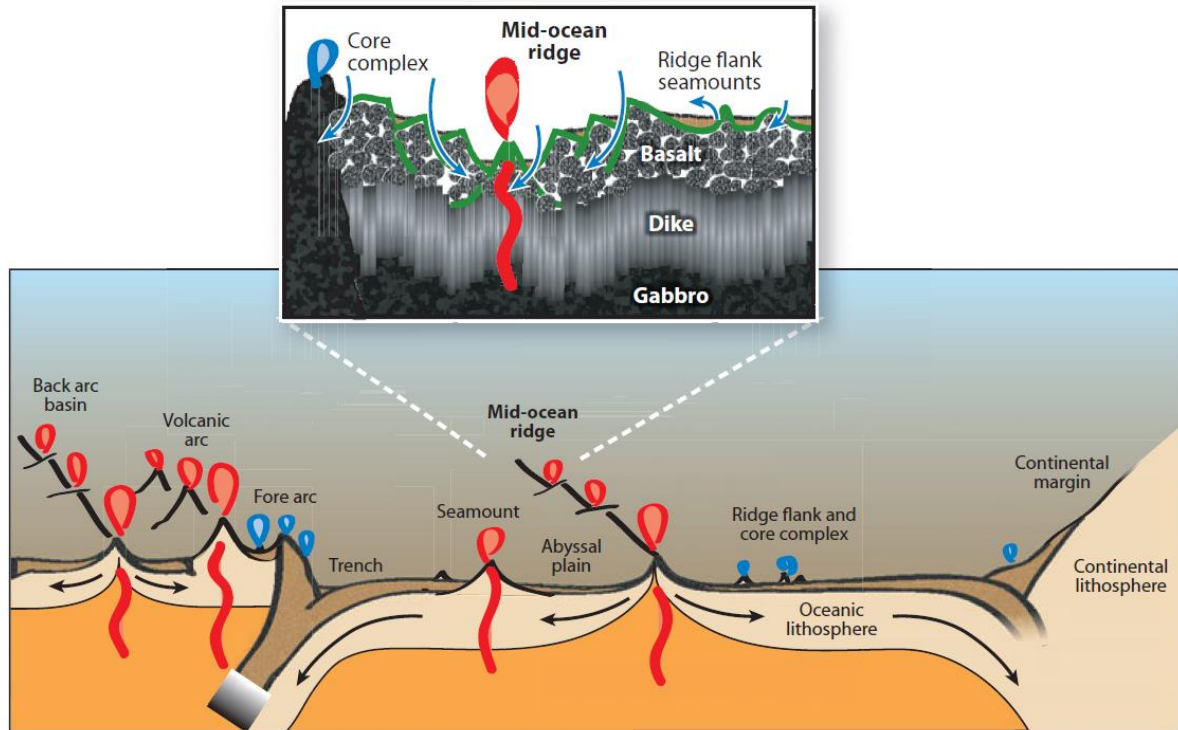


Figure 0.1: Global distribution of hydrothermal venting. S. Beaulieu, K. Joyce, and S.A. Soule (WHOI), 2010, <http://www.interridge.org/irvents/maps>. Funding from InterRidge and Morss

Colloquium Program at WHOI.

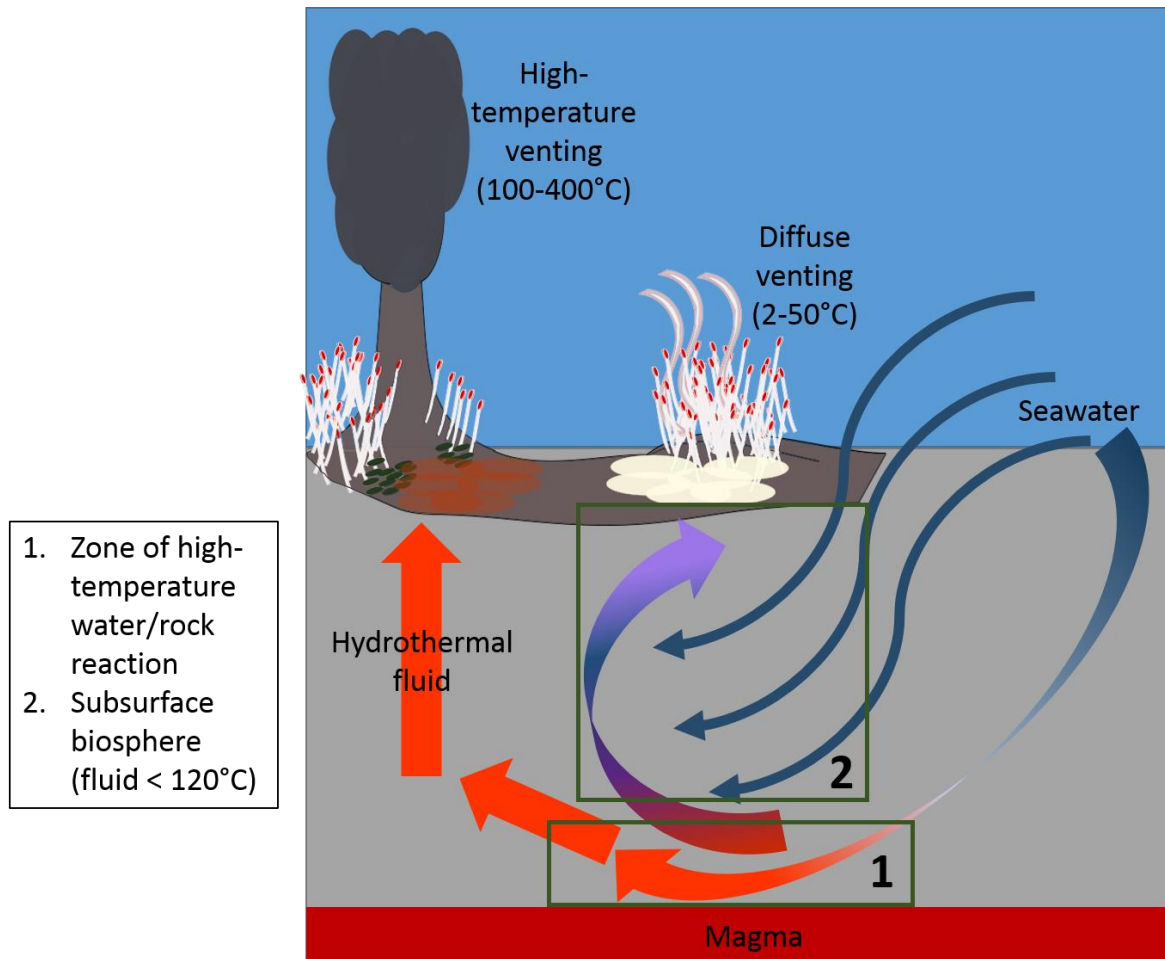


**Figure 0.2: Volcanism and hydrothermal venting on the seafloor**(Schrenk *et al.*, 2010).

Unlike terrestrial hot springs, however, the hydrothermal fluid in deep-sea systems can be heated up to 400°C, as the hydrostatic pressure of the ocean above forces it to stay liquid. The chemical disequilibrium created by the mixture of hot, reducing, metal-heavy hydrothermal fluid and cold, oxidizing, metal-poor seawater (Edwards *et al.*, 2011) at the point where this mixed fluid emerges from the seafloor creates “chimneys” of metallic sulfide deposits as metals and sulfides precipitate out (Figure 1.3). The precise chemistry of hydrothermal fluid within a venting system is primarily controlled by phase separation – as hydrothermal fluid is altered at depth, brines (high-salt fluids) segregate out, creating hydrothermal fluids with significant differences in chlorinity and other solute composition within the same hydrothermal field (Butterfield *et al.*, 1994). There are also major differences between the chemical composition of hydrothermal fluids from basaltic



mid-ocean ridge spreading centers, back-arc volcanic zones, and areas of serpentinization such as the Lost City vent field (Schrenk *et al.*, 2004), where water/rock reactions abiotically produce hydrogen and methane.



**Figure 0.3: Diagram illustrating the alteration of seawater to hydrothermal fluid via water/rock reactions in the heated crustal zone near upwelling magma. The hot, reducing, metal-rich hydrothermal fluid can either return to the ocean floor directly and precipitate out metal-sulfide ‘chimneys’ as it emerges into the cold, oxic ocean, or it can be progressively diluted by unaltered seawater and emerge as low-temperature, ‘diffuse’ hydrothermal fluid. Subsurface hydrothermal fluid/seawater mixtures below approximately 120°C host the subsurface biosphere.**

The energy from this geochemical flux is utilized by chemolithoautotrophs both below and above the seafloor, forming the base of a food web that eventually supports macro-organic life, such as crabs and tubeworms (Corliss *et al.*, 1979). Deep-sea hydrothermal vents, apart from supporting a biosphere on the seafloor, also serve as windows to the subsurface biosphere, the 10-20% of organic carbon on Earth thought to be contained in prokaryotes living in the oceanic crust (Whitman *et al.*, 1998). The concept of vents as “windows to the subsurface” was first suggested in the context of high-temperature venting (Deming and Baross, 1993) but is probably more applicable to low-temperature venting fluids which are within the known range for life (up to 122°C) (Takai, Nakamura, *et al.*, 2008). Very low-temperature fluids are referred to as “diffuse fluid”, and have circulated through the crust and been diluted with entrained seawater down to temperatures of 2-50°C at the venting point, more than cool enough to harbor life (Summit and Baross, 2001). The study of the geochemistry and microbiology of these fluids helps us to constrain how much of Earth’s carbon may be contained in the biosphere of the oceanic crust, and how it is being cycled by the organisms that live there. The subsurface biosphere at hydrothermal vents may extend as far as several hundred meters into the crust but its extent is largely unconstrained (Amend and Teske, 2005; Stevens, 1997).

Furthermore, deep-sea hydrothermal vents are useful analogue environments to potential habitats on Mars, Europa, and the early Earth. Evidence has been found of hydrothermal venting on Mars through volcanism and/or meteor impacts (Griffith and Shock, 1997; Ehlmann *et al.*, 2011). Europa’s proposed habitable environment is an iced-over ocean heated by a molten core (Rothschild and Mancinelli, 2001; Des Marais *et al.*, 2008). In

this case, hydrothermal venting would be one of the only viable sources of energy.

Hydrothermal vents are also suggested as a likely site for the origin of life on early Earth, which had a strongly reducing, geologically active environment (Baross and Hoffman, 1985). This makes the study of hydrothermal vent sites very useful to understanding potential extraterrestrial and origin-of-life habitats, in terms of both their seafloor and subseafloor biota.

### 1.3.2 Axial Volcano

The field site for the work in this thesis is Axial Volcano, located approximately 500 km off the coast of the Pacific Northwestern United States and approximately 1.5 km deep, on the Juan de Fuca Ridge (Figure 1.4). First discovered and mapped using the deep-sea research submarine *Alvin* in the early 1980s, it has been the site of active, long-term hydrothermal venting, as well as several eruptions during the last thirty years (Huber *et al.*, 2003; Embley *et al.*, 1999; Chadwick *et al.*, 2012). It is currently the site of efforts to develop a permanent, cabled observatory returning real-time geochemical and physical data from venting sites to scientists on land (Delaney *et al.*, 2001; Kelley *et al.*, 2014).

Axial Volcano has a complex geologic history (Clague *et al.*, 2013). It is located on a mid-ocean spreading center, the Juan de Fuca Ridge, and is the dominant local feature, rising approximately 1400 m above the seafloor. Its volcanism and hydrothermal activity results from the interaction of the spreading center and the Cobb-Eickelberg Seamount Chain hotspot (Johnson and Embley, 1990), making it geologically unusual and subsequently well-studied, given its shallow depth and proximity to a number of ports on the western coast of North America.

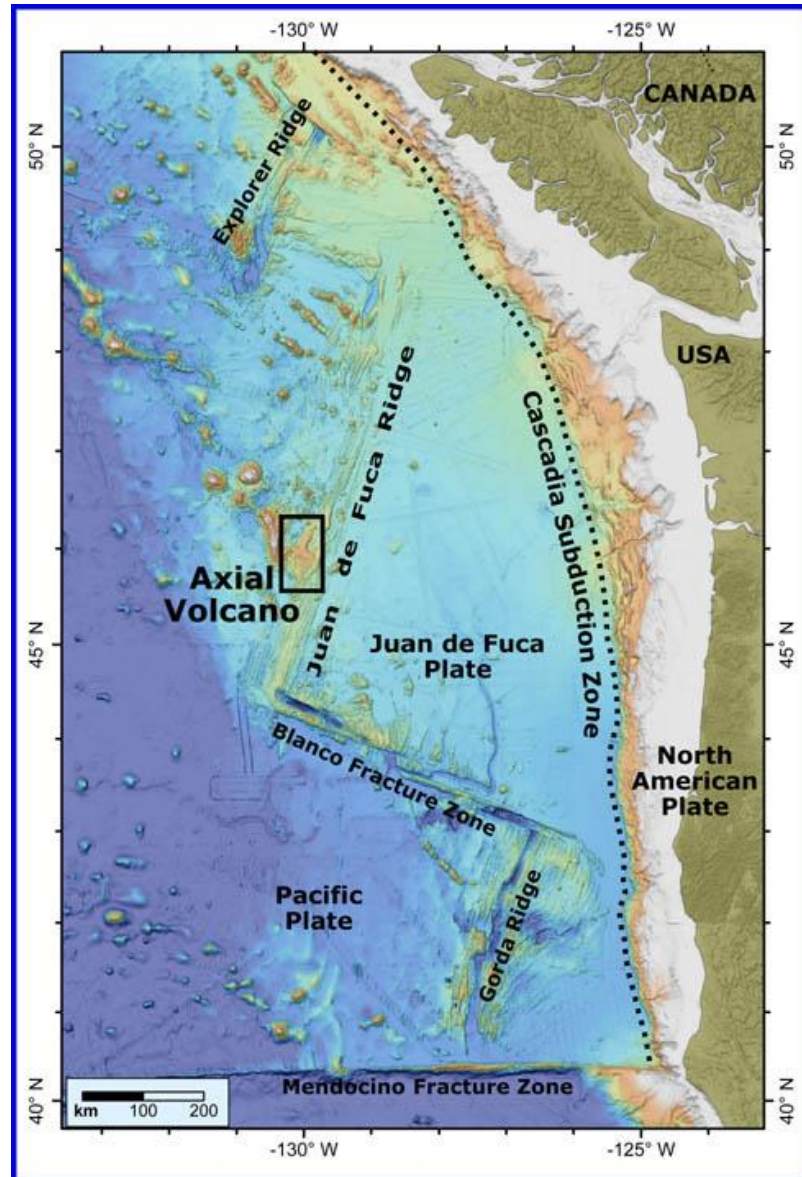


Figure 0.4: Location of Axial Volcano and the Juan de Fuca Ridge ([www.pmel.noaa.gov/eoi/nemo](http://www.pmel.noaa.gov/eoi/nemo)).

Axial has a horseshoe-shaped caldera, approximately 30,000 years old, which has erupted with a roughly 13-year period for the last 800 years, the location of lava flows shifting around the caldera over time (Clague *et al.*, 2013). Two of the last three eruptions have both occurred at the southern end of the caldera (Embley *et al.*, 1999; Chadwick *et al.*,

2012) which is also the location of three active hydrothermal vent fields associated with Axial.

The eastern rim of the caldera hosts the main field, known as the International District, which is less than 400 years old, and the other vent fields are even younger, probably less than 100 years. Unlike the nearby Endeavour Segment hydrothermal vent fields, sulfide chimneys are rare at Axial and only prominent in the International District. The ASHES (Axial Submarine Hydrothermal Event Survey) vent field on the western side of the caldera consists largely of diffuse (2-50°C) venting and anhydrite mounds, with three metal-sulfide edifices named Hell, Inferno and Mushroom. The vents north of the International District are also diffuse. The two historical eruptions observed created a number of “snowblower” vents, areas of diffuse venting heavy with white sulfur floc, presumably from sulfur-oxidizing organisms in the subsurface, which have no point source and largely disappear within a year or two of the eruption as the fresh hydrothermal fluid which sustained them is exhausted (Meyer *et al.*, 2013). Other vents have been paved over by lava flows during the eruption. However, several individual vents have been extant for most of the ~30 year observational history of Axial Volcano and are the subject of time-series measurements of their geochemistry and biology. In particular, our studies have focused on the diffuse vents Marker 113 on the western end of the eastern vent field, Marker 33 on the northern end of the eastern vent field, and the diffuse vent Anemone in the ASHES field. Marker 113 is considered a “hotspot” for microbiological activity and high in methane, and Anemone has experienced wide fluctuations in available hydrogen, presumably as the subsurface plumbing of the hydrothermal field changes during the eruptive cycle (unpublished data).

The hydrothermal vents at Axial are host to a complex microbiological community. Most-probable-number (MPN) surveys over several years have shown the consistent presence of thermophilic and hyperthermophilic methanogens and anaerobic sulfur-reducing heterotrophs, as well as small numbers of anaerobic autotrophic iron-reducers and sulfur/thiosulfate-reducing bacteria (Holden *et al.*, 1998; Huber *et al.*, 2002, 2003; Ver Eecke *et al.*, 2012). Molecular surveys have found evidence of hydrogenotrophic (hyper)thermophilic methanogens at multiple vent sites (Meyer *et al.*, 2013; Opatkiewicz *et al.*, 2009; Ver Eecke *et al.*, 2012) as well as hyperthermophilic heterotrophs and thermophilic sulfur-reducing bacteria (Huber *et al.*, 2003). The dominant mesophilic group are sulfur-oxidizing epsilonproteobacteria (Huber *et al.*, 2003, 2007; Meyer and Huber, 2014).

### 1.3.3 High Rise Field, Endeavour Segment

The Endeavour Segment is another venting site on the Juan de Fuca Ridge. It was first identified in the 1980s (Robigou *et al.*, 1993) and has been continuously studied since. It has five main venting fields (Delaney *et al.*, 1992) which have distinctly different geochemical characteristics: Sasquatch, Salty Dawg, High Rise, Main Endeavour Field, and Mothra. Unlike Axial Volcano, it is not associated with active volcanism and appears to be a geologically “mature” venting system (Butterfield *et al.*, 1994). Geochemically, the Endeavour Segment is very different as well; fluid vented at Endeavour has a much higher pH, high concentrations of methane, and very low concentrations of hydrogen (Lilley *et al.*, 1993; Butterfield *et al.*, 1994). Mass spectroscopy suggests that some diffuse fluids at Endeavour are depleted of hydrogen by methanogens growing in the

subsurface hydrothermal system, which also produces some of the methane observed (Wankel *et al.*, 2011).

Most hydrothermal venting at Endeavour is associated with spectacular sulfide chimneys up to 40 m high (Robigou *et al.*, 1993) with areas of diffuse flow found between the main venting fields, which are spaced in a line north to south along the main segment (Kelley *et al.*, 2014). The High Rise field at Endeavour is an area approximately 350 m by 150 m containing 10 large actively venting sulfide structures, which form the core of the field (Robigou *et al.*, 1993). The distinctive feature of these sulfide structures is their “flanges”, large horizontal outcrops which cause hydrothermal fluid to pool beneath them, creating an environment which hosts an associated biota including tubeworms and other macrofauna (Delaney *et al.*, 1992).

Hydrogenotrophic organisms have been detected even at low-hydrogen, high-temperature venting sites at Endeavour (Ver Eecke *et al.*, 2009, 2012; Lin *et al.*, 2014). They may be syntrophically supported by hydrogen-producing hyperthermophilic heterotrophs, a process observed both at hydrothermal vents and in other subsurface environments with low H<sub>2</sub> concentrations (Ver Eecke *et al.*, 2012; Davidova *et al.*, 2012; Schopf *et al.*, 2008). Organisms found in culture-based surveys of diffuse fluid include hyperthermophilic iron-reducers, methanogens, and heterotrophs (Ver Eecke *et al.*, 2009, 2012). Culture-independent surveys of diversity in sulfides and diffuse flow fluid at Endeavour have identified groups including thermophilic and hyperthermophilic iron-reducers, hyperthermophilic heterotrophs and methanogens, mesophilic sulfate- and sulfur-reducers, and mesophilic sulfur-oxidizers (Lin, 2014; Anderson *et al.*, 2015; Schrenk *et al.*, 2003).

## 1.4 Hyperthermophilic Anaerobes in Hydrothermal Vent Systems and the Subsurface Biosphere

### 1.4.1 Molecular Surveys of Hyperthermophile Diversity

Hydrothermal vent sites are hotspots of biological activity in the deep ocean, and molecular surveys of microbial diversity show a wide range of organisms. In general, abundant groups (defined as those comprising >1% of a community) tend to be widespread and found at multiple types of vent sites, whereas rare groups (<0.1% of a community) are more geographically restricted (Anderson *et al.*, 2015). However, it is also true that for archaeal diversity in particular, 16S rRNA gene sequences do not adequately represent true phenotypic diversity, with many archaeal hyperthermophiles having 16S rRNA gene sequences which are >97% similar (above the cutoff for defining novel species) while being phenotypically dissimilar (Holden *et al.*, 2001).

The groups of organisms most consistently seen across hydrothermal vent sites are those that are actually found in seawater (diffuse hydrothermal fluid containing a high seawater component) such as the crenarchaeotal Marine Groups I and II, which can easily travel between vent sites (Anderson *et al.*, 2015). Also abundant and widespread are mesophilic sulfur-oxidizers of the Epsilonproteobacteria, as the steep chemical gradient between sulfide-rich hydrothermal fluid and oxic seawater provides an abundant source of energy for this particular form of chemoautotrophy (Nunoura and Takai, 2009). However, sulfur oxidation requires oxygen and is most favorable in mesophilic environments (McCollom and Shock, 1997), and is not representative of the anaerobic subsurface, although microaerophilic sulfur-oxidizers such as *Arcobacter* are probably important in the near-surface, highly dilute fluid (Meyer *et al.*, 2013). In order to understand diversity in the



subsurface, we must confine our examination to sequences belonging to thermophilic and hyperthermophilic groups. Sequences from thermophilic and hyperthermophilic organisms, on the other hand, when found in diffuse fluid, can be taken to indicate the community composition of the high-temperature anaerobic subsurface communities in hydrothermal vent systems (Stevens, 1997; Holden *et al.*, 1998; Butterfield *et al.*, 1998; Summit and Baross, 2001; Amend and Teske, 2005).

Some archaeal thermophilic and hyperthermophilic groups are found wherever the geochemistry of the vent site is favorable for their metabolisms; these include the *Methanococcales* and *Methanopyrales* (hydrogenotrophic, autotrophic methanogens, both thermophilic and hyperthermophilic), the *Archaeoglobales* (hyperthermophilic, hydrogenotrophic, autotrophic sulfur and iron reducers), *Thermococcales* (hyperthermophilic heterotrophs, facultative sulfur-reducers), and *Desulfurococcales* (hyperthermophilic, hydrogenotrophic and heterotrophic sulfur and iron reducers) (Nakagawa *et al.*, 2006; Takai, Nunoura, *et al.*, 2008). Sequences representing the single hyperthermophilic group of the Bacteria, the *Thermotogae*, have been found at some vent sites (Takai *et al.*, 2008; Huber *et al.*, 2006). There are a much wider variety of thermophilic bacterial sequences in diffuse fluid. These include sequences from the phylum *Aquificae*, the family *Nautiliaceae*, the order *Thermales*, and the phylum *Thermodesulfobacteria* (Orcutt *et al.*, 2011). The *Aquificae* are autotrophic hydrogen-oxidizers and/or nitrate- and sulfur-reducers, and are found frequently in vent systems (Huber and Holden, 2008). The *Nautiliaceae* are hydrogen-oxidizing nitrate and/or sulfur-reducers, obligately or facultatively anaerobic, and have been isolated exclusively at hydrothermal vent sites (Nakagawa and Takai, 2014). The *Thermales* genera found at

vents are microaerophilic or aerobic heterotrophs such as *Oceanithermus*, *Marinithermus*, and *Rhodothermus* (Takai *et al.*, 2008; Takai *et al.*, 2009; Perner *et al.*, 2007; Nakagawa *et al.*, 2005). The *Thermodesulfobacteria* are anaerobic, thermophilic sulfate-reducers and can be autotrophic or heterotrophic (Jeanthon *et al.*, 2002). The consistent theme of the thermophilic and hyperthermophilic genera found in molecular surveys of hydrothermal vents is that the exclusively thermophilic and hyperthermophilic genera have cultivated members which are all anaerobes. They have metabolisms which rely on hydrogen and sulfur, or more infrequently nitrate and iron. The most common link is hydrogen; most autotrophic anaerobes can or must use it as an electron donor, and many heterotrophs produce it as a waste product. This thesis focuses on two key groups of hydrogenotrophs at hydrothermal vents: methanogens and sulfur-reducers.

#### 1.4.2 Thermophilic and Hyperthermophilic Methanogens

Methanogens form a monophyletic group in the archaeal phylum *Euryarchaeota* (Gao and Gupta, 2007). Utilizing the Wood-Ljungdahl or acetyl-CoA pathway, they obtain energy by producing methane, either through splitting acetate and reducing the methyl group, or reducing carbon dioxide with electrons from hydrogen, formate, or carbon monoxide, as well as occasionally from other methyl groups (Ferry, 2010). There are no known facultative methanogens. The key enzyme for methanogenesis, methyl coenzyme M reductase (coded for by the *mcr* genes, which are unique to methanogens), operates only under strictly anaerobic conditions (Ermler, 2005). All methanogens are strict anaerobes and most are highly sensitive to oxygen, requiring a reduced (<-300 mV) as well as anoxic environment. They are found in a diverse range of anoxic environments, including wetland soils, animal rumens and digestive tracts, subsurface terrestrial

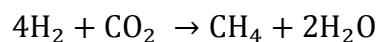
environments, anthropogenic anoxic environments such as sewage reactors, and deep-sea hydrothermal vents (Whitman *et al.*, 2006). Different groups are adapted to a wide range of temperatures and salinities, but pH optima are generally mildly acidic to mildly alkaline.

Methanogens are responsible for the production of the vast bulk of methane on Earth, although abiogenic production of methane has also been observed (Foustoukos and Seyfried, 2004). Methanogenesis is a barely energetically favorable metabolic strategy compared to other autotrophic metabolisms, and it is usually only favored when alternative electron acceptors are not available or have been consumed, so methanogens live on the very limits of life wherever they are (Valentine, 2007; Deppenmeier and Müller, 2008). It has been suggested that methanogenesis could have been the metabolism for the Last Universal Common Ancestor (LUCA) or other early autotrophs, as it is favored in the reducing environment thought to have been present on the early Earth (Lane *et al.*, 2010).

Several genera of methanogens are entirely thermophilic or hyperthermophilic. The hyperthermophilic methanogens are found in the genera *Methanothermus*, *Methanocaldococcus*, *Methanotorris*, and *Methanopyrus*. *Methanothermus* is the only terrestrial genus of hyperthermophilic methanogens, and includes two species isolated from solfataric fields with temperature optima of 83°C and 88°C (Stetter *et al.*, 1981; Lauerer *et al.*, 1986). The other three hyperthermophilic methanogen genera are all marine, found at hydrothermal vent systems. The cultivated members of the *Methanocaldococci* are exclusively hyperthermophiles with temperature optima from 80 to 90°C, circumneutral pH optima, and salinity optima approximating seawater (Whitman

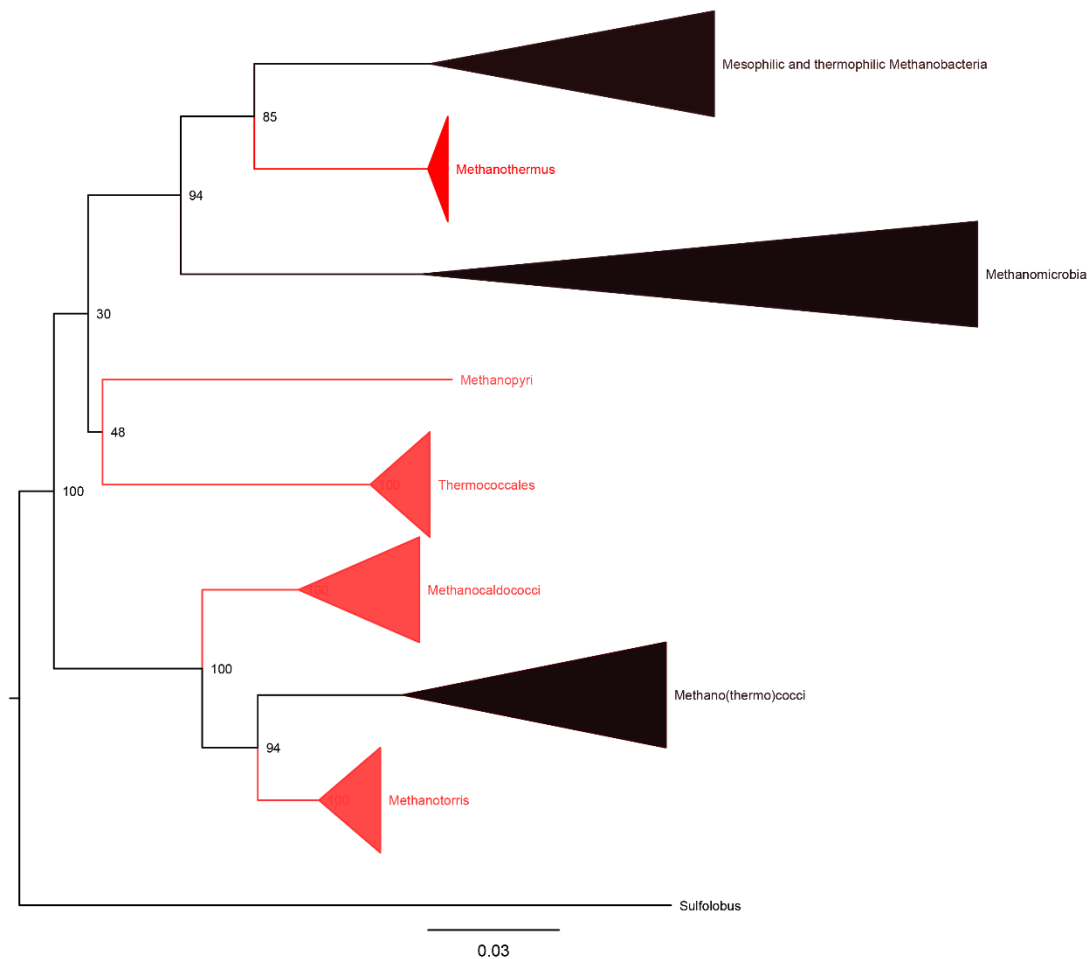
and Jeanthon, 2006). *Methanocaldococcus jannaschii* is the best-studied of the hyperthermophilic methanogens and was the first characterized (Jones *et al.*, 1983). *Methanocaldococcus* sp. FS406-22 is notable for its ability to fix nitrogen at 92°C, the current upper temperature limit for biological nitrogen fixation (Mehta and Baross, 2006). The genus *Methanopyrus* has one cultivated member, *Methanopyrus kandleri*, which has the highest optimum temperature of any cultivated methanogen at 98°C and can grow at temperatures of up to 110°C (Kurr *et al.*, 1991). The two cultivated species of the genus *Methanotorris* have temperature optima of 75°C and 88°C (Takai *et al.*, 2004; Burggraf *et al.*, 1990) and *Methanotorris igneus* is the most acidophilic thermophilic methanogen with a pH optimum of 5.7 (Burggraf *et al.*, 1990).

The hyperthermophilic methanogens are all strict hydrogenotrophs, relying solely on methanogenesis via the consumption of hydrogen and carbon dioxide, as in the following equation:



Thermophilic methanogens are found in a number of genera which include both mesophiles and thermophiles; notable ones are *Methanothermobacter* and several species within the genus *Methanobacterium* (Whitman *et al.*, 2006). Most can grow using either H<sub>2</sub> or formate as electron donors, unlike the *Methanocaldococci*. The only strictly marine thermophilic genus is *Methanothermococcus*, which is most closely related to the *Methanocaldococci* and *Methanococci*. *Methanothermococcus thermolithotrophicus* is capable of fixing nitrogen when given nitrogen gas as its only nitrogen source (Huber *et al.*, 1982). The genus *Methanococcus* largely consists of mesophiles, but one member,

*Methanococcus aeolicus*, grows at mildly thermophilic temperatures (optimum 46°C, range 20-55°C) (Kendall *et al.*, 2006). The methanogens generally have 16S rRNA gene sequences with very high sequence similarity, meaning that it is sometimes necessary to use the *mcrA* gene (found only in methanogens) to distinguish their phylogenetic relationships (Luton *et al.*, 2002).



**Figure 0.5: Phylogenetic tree of the methanogens, also showing relationship to the Thermococcales. Created using the Ribosomal Database Project Tree Builder (<http://rdp.cme.msu.edu/treebuilder/treeing.spr>). Hyperthermophilic groups are in red. Bar, 3% sequence divergence.**

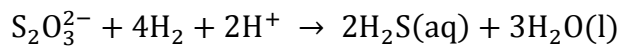
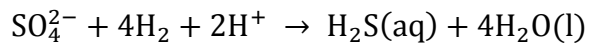
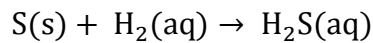
### 1.4.3 Thermophilic Sulfur Reducers

Another group of thermophilic, hydrogenotrophic organisms are the sulfur-reducers.

Unlike methanogens, the sulfur-reducing bacteria and archaea are a functional group rather than a monophyletic one. Sulfur-reduction as a metabolic strategy encompasses the use of sulfur-containing compounds as electron acceptors for both heterotrophic (where the electron donor is organic matter) and autotrophic (where the electron donor is most often hydrogen) organisms. The sulfur compounds can be sulfate, sulfite, thiosulfate, or elemental sulfur. Unlike methanogens, sulfur-reducing prokaryotes are usually capable of multiple metabolisms. Sulfur-reducing autotrophs can often reduce nitrate or iron, and sulfur-reducing heterotrophs can also be capable of transferring electrons to protons, creating molecular hydrogen (H<sub>2</sub>).

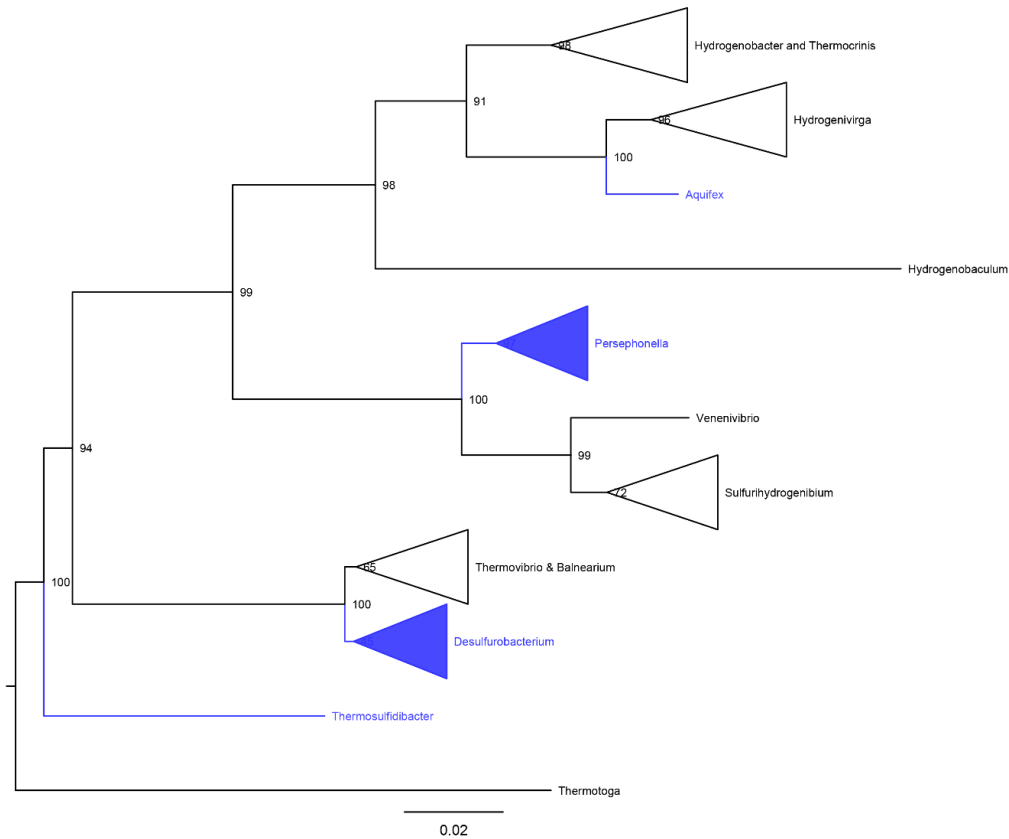
Sulfate-reducing bacteria (SRB) are responsible for the vast majority of biogeochemical sulfur cycling, and are found in the Deltaproteobacteria (the majority of SRB genera), the Gram-positive bacteria (the genera *Desulfotomaculum* and *Desulfosporosinus*) and the genera *Thermodesulfobacterium* and *Thermodesulfovibrio*. Only the last two are exclusively thermophilic (Rabus *et al.*, 2006). There is only one known genus of sulfate-reducing archaea, *Archaeoglobus*, all members of which are hyperthermophilic and all but one of which are facultatively chemolithoautotrophic. The sole known exception is an obligate mixotroph (Dahl and Truper, 2001). *Archaeoglobus* sequences have been detected in hydrothermal vent settings (Nercessian *et al.*, 2003; Opatkiewicz *et al.*, 2009; Huber *et al.*, 2006; Nakagawa *et al.*, 2005) as have the exclusively thermophilic *Thermodesulfobacteria* (Nercessian *et al.*, 2005; Nakagawa *et al.*, 2006).

Sulfur-reducing organisms are defined as those which cannot reduce sulfate, but are still capable of reducing other sulfur-containing compounds, principally elemental sulfur and two of the intermediate products of sulfate reduction, thiosulfate and sulfite (which can also be produced abiotically from sulfide oxidation (Moses *et al.*, 1987; Schippers *et al.*, 1996)). These organisms can reduce sulfur, sulfate, and/or thiosulfate to sulfide using hydrogen or organic electron donors, as in the following equations:



They can also be facultative anaerobes, capable of using oxygen as a terminal electron acceptor when it is available, although most are strictly anaerobic (Rabus *et al.*, 2006). The sulfur-reducers are found across a range of phylogenetic groups in both bacteria and archaea. In the context of hydrothermal vent systems, the most important thermophilic sulfur-reducing bacteria are the *Aquificales*, in particular the genus *Desulfurobacterium*. The *Aquificales* are mostly hydrogen oxidizers, but some can reduce nitrate and sulfur as well (Huber and Eder, 2006). The *Desulfurobacteria* are strictly marine, anaerobic, chemolithoautotrophs which oxidize hydrogen and reduce elemental sulfur, thiosulfate, and/or nitrate (L'Haridon *et al.*, 2006). All the cultivated strains of this genus come from deep-sea hydrothermal vent systems, and they appear to be widespread. They have temperature optima between 60 and 75°C, temperature ranges from 40-80°C, circumneutral pH optima and ranges, and salinity optima around that of seawater,

although most can tolerate up to twice the salinity of average seawater (L'Haridon *et al.*, 2006).



**Figure 0.6: Phylogenetic tree of the phylum Aquificae. Created using the Ribosomal Database Project Tree Builder (<http://rdp.cme.msu.edu/treebuilder/treeing.spr>). Groups associated with deep-sea hydrothermal vent systems, including the *Desulfurobacteria*, are in blue. Bar, 2% sequence divergence.**

Autotrophic, hyperthermophilic sulfur-reducers are rarer, represented at hydrothermal vent systems only by the archaeal genus *Archaeoglobus*, which, as mentioned previously, can also reduce sulfate. The archaeal genera *Thermoproteus*, *Thermococcus*, *Pyrococcus*, and *Pyrobaculum* can reduce elemental sulfur heterotrophically, and all are found at hydrothermal vent systems (Orcutt *et al.*, 2011).



#### 1.4.4 Other Hyperthermophilic Organisms at Hydrothermal Vents

Hyperthermophilic iron-reducers are found at hydrothermal vents, and can compete with methanogens for hydrogen. While the reduction of iron(III) to iron(II) is a well-studied metabolism at mesophilic temperatures, hyperthermophilic iron reduction was only discovered approximately fifteen years ago (Vargas *et al.*, 1998). There are still only a few cultivated organisms which have been demonstrated to reduce iron directly as a source of energy, as opposed to indirectly reducing it by producing organic metabolites which reduce iron. Hyperthermophilic iron reducers associated with hydrothermal vents include the crenarchaeotal genera *Pyrobaculum*, *Pyrodictium*, and *Hyperthermus* (Takai and Sako, 1999; Ver Eecke *et al.*, 2009; Lin *et al.*, 2014). Some are facultatively heterotrophic or can use nitrate as an alternative electron acceptor. As they primarily use insoluble iron compounds as electron acceptors, these organisms are most closely associated with iron sulfide structures (where the abiotic oxidation of iron sulfides provides them with substrates) rather than diffuse hydrothermal venting.

As mentioned briefly above, hyperthermophilic heterotrophic sulfur-reducers, notably the euryarchaeotal genera *Thermococcus* and *Pyrococcus*, are frequently found in hydrothermal vent systems. They can interact with other prokaryotes in two possible ways. In the first and simplest, they can be living on organic debris from autotrophs, effectively forming a second trophic layer (Shock and Holland, 2004). While these organisms can use elemental sulfur as an electron acceptor, in its absence they will use protons, producing molecular hydrogen as a byproduct. As in mesophilic systems, this has been demonstrated in laboratory cultures to support hydrogenotrophic hyperthermophiles such as methanogens when the organisms are grown in co-culture

(Ver Eecke *et al.*, 2012). In this mode hydrogen-using autotrophs would not be primary producers unless the organic matter the heterotrophs were growing on was *also* produced via autotrophic primary production at the vent site.

## 1.5 Modelling of Hydrothermal Vent Systems

### 1.5.1 Prior Geochemical Modelling of Habitability at Vent Systems

Over the years, several models have been constructed using geochemical data from hydrothermal vents to model the potential distribution of autotrophic microorganisms based on relative availability of Gibbs reaction energies ( $\Delta G_r$ ) to different modes of metabolism. The  $\Delta G_r$  of a reaction is generally expressed in such models in kJ per kilogram of vent fluid, based on the concentration of the specific substrates and products in unmodified fluid and the overall chemical composition. Many autotrophs compete for substrates – for example, the various hydrogenotrophic autotrophs such as iron-reducers, sulfur-reducers, and methanogens – so relative  $\Delta G_r$  is important in determining habitability at any given spot for each type of metabolism.

The first major model of this type was presented by McCollom and Shock (1997), which predicted the energetic favorability of autotrophic metabolisms over a range of temperatures, based on the fluid composition of a vent at the East Pacific Rise. They calculated  $\Delta G_r$  values using the following equation:

$$\Delta G_r = \Delta G^\circ + RT \ln Q$$

$\Delta G^\circ$  is the *standard* Gibbs energy, R is the universal gas constant, T the temperature (K) and Q the activity product of the species involved in the reaction. This adjusted the available Gibbs energies for the unusual and often extreme conditions of the vent

environment. Over the range of temperatures at vent environments conducive to life (2°C - ~120°C) they concluded that anaerobic metabolisms such as methanogenesis, iron reduction, and sulfur reduction would be favored at thermophilic and hyperthermophilic temperatures (>38°C) while aerobic metabolisms such as sulfur oxidation would be favored at mesophilic temperatures. The primary restrictions on this model were the suppression of mineral precipitation (which is clearly in effect at vents producing sulfide chimneys) and the assumption of equilibrium for the “knallgas” reaction ( $\text{H}_2 + \frac{1}{2}\text{O}_2 \rightarrow \text{H}_2\text{O}$ ), although cultures of microaerophilic bacteria metabolizing using this reaction have since been grown from hydrothermal vent samples (Takai, Gamo, *et al.*, 2004; Reysenbach *et al.*, 2000). Tivey (2004) investigated the energetic favorability of sulfur and methane-based metabolisms in a sulfide system (Endeavour) where diffusion and advection would have strong effects on mixing, and predicted much lower-temperature transitions from aerobic to anaerobic metabolisms (9-38°C) but reported similar  $\Delta G_r$  values for methanogenesis, sulfur reduction, sulfide oxidation, and methanotrophy. Houghton and Seyfried (2010) combined experimental studies of fluid-mineral reactions under hydrothermal vent conditions with calculations of energetic availability, comparing energy availability between nascent and mature sulfide chimney structures and with microbial community composition and richness in those sulfides. They found suggestive but not definitive links between the geochemical energy availability and the community composition. Dahle *et al.* (2015) modelled predicted microbial metabolisms based on limiting substrates for their predicted energetically favorable metabolisms, based on Gibbs energies, and compared them with microbial community composition derived from 16S rRNA gene sequences.

The major piece of data lacking from all these models, however, is a physiologically-based estimate of microbial energetic requirements under the prevailing conditions at hydrothermal vents. McCollom and Shock use an estimate of the minimum energetic requirement for ATP production from Thauer et al. (1977). Tivey, Houghton and Seyfried, and Dahle et al. do not pinpoint a specific minimum energetic requirement for a metabolism to support chemoautotrophic growth beyond being energetically favorable overall (i.e. having a negative  $\Delta G_r$ ). Without culture-based determinations of microbial substrate and energy requirements, it is not possible to accurately determine the minimum energy availability that supports any given microbial metabolism. So what is a minimum microbial energy requirement, and how can it be measured?

#### 1.5.2 Microbial Maintenance Energies

Biological energy minima can be considered in two ways – the biological energy quantum and maintenance energy requirements (Hoehler, 2004). The biological energy quantum (BEQ) is the concept that to make one molecule of ATP, a cell must have a certain energy flux available to it, which functions as a minimum energy requirement (Hoehler, 2004). Cells with different energy-generating metabolisms will have different BEQs, depending on how many molecules of ATP they generate with each electron transfer from their electron donor. This may cause their maintenance energy to differ from cells with similar energy requirements in terms of cellular maintenance but more efficient modes of energy generation. The maintenance energy requirement, on the other hand, is the *flux* of energy required to sustain the organism. Maintenance energies for prokaryotes can be broadly divided into three categories; “survival energy”, or rates of energy usage needed to prevent a cell degrading without supporting an active

metabolism; “maintenance energy”, or the rate of energy usage minimally needed to sustain a cell’s metabolic activity without growth; and “growth energy”, or rates of energy usage minimally needed for a cell to reproduce (Morita, 1999). These concepts should be thought of less as specific values and more as *ranges* of energy usage. There are clearly a wide range of energy usage rates that support growth – the higher the rate, the faster the rate of growth, until the organism reaches its maximum growth rate. Less clearly, there should also be a range of energy usage rates that can be considered “maintenance energy” – from just below the amount of energy required for the cell to replicate, to barely enough energy for the cell to metabolize. The single requisite function for “survival energy” is to repair DNA damage in order that the cell can create proteins accurately once it begins to metabolize actively again, but this is the only true minimum value.

A meta-analysis of microbial maintenance energy measurements (Tijhuis *et al.*, 1993) (in this case “true” maintenance energies, the measurement that permits assessment of whether a cell can survive actively in an area of given energy flux) suggested that they are related to organisms’ temperature optima, rising with rising temperature. However, this meta-analysis is not necessarily applicable to chemoautotrophs in anaerobic vent environments; it lacks maintenance energy data for organisms at high thermophilic and hyperthermophilic temperatures, especially anaerobes. Only five anaerobic measurements included in the Tijhuis *et al.* meta-analysis are below or above the mesophilic 30-40°C range. Moreover, the majority of the data was gathered from studies with other aims where energetic data was a byproduct, or industrial studies where the focus was on optimizing production, rather than energetic requirements under environmental

conditions – for instance, the studies on *Methanothermococcus thermolithotrophicus*, which provide two of the three data points for thermophilic anaerobes (Fardeau and Belaich, 1986; Peillex *et al.*, 1988). Anaerobic thermophiles and hyperthermophiles form an important part of hydrothermal vent ecology and a large percentage of autotrophs at vent sites (Kimura *et al.*, 2007; Huber *et al.*, 2006; Ver Eecke *et al.*, 2009; Huber *et al.*, 2003).

The other important question raised is whether temperature should be the only environmental variable considered when estimating energy requirements. Temperature is not the only environmental stressor which requires expenditure of energy in order for cells to survive; acidophiles must constantly pump out protons in order to maintain their internal pH, among other examples (Hoehler, 2007). A study of growth energies in hyperthermophilic methanogens suggested they were species-dependent and well as temperature-dependent (Ver Eecke, 2011). Other work has shown that maintenance energies can increase with stressors such as substrate inhibition (Chen and Johns, 1996). Other potential factors affecting a cell's maintenance energy could be total cell size; size of the genome; number of genome copies; and other environmental stress-causing constraints such as pH, temperature, or salt concentration (Valentine, 2007).

### 1.5.3 Reactive Transport Flow Modelling and Modelling Parameters

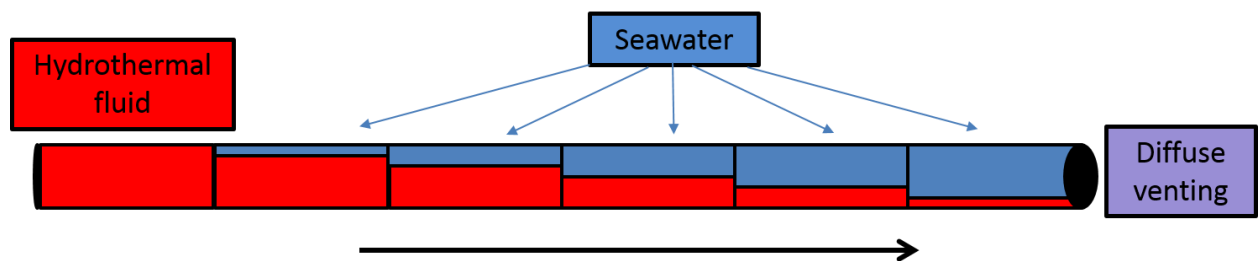
It is evident that the majority of previous modelling of hydrothermal vents has focused on directly comparing Gibbs energies for a mixed parcel of hydrothermal fluid and seawater (the mixing ratio determined by temperature) with theoretically derived maintenance energies and assuming that metabolisms with energies greater than maintenance energy will be possible at that particular mixing ratio/temperature. This approach allows the

prediction of microbial distribution – which can be tested by looking at molecular diversity, as in Dahle et al. (2015) – but it is not particularly useful for understanding *processes* in the hydrothermal subsurface. Modelling of terrestrial subsurface biospheres has largely focused on qualifying and/or quantifying *rates* of key biogeochemical processes. These are most commonly examined through reactive transport modelling/models (RTM(s)).

The most famous joke about modelling involves a physicist who is asked to calculate the amount of leather available in a cow's hide, and begins "Consider a spherical cow..." (Harte, 1988). Although entirely apocryphal, it tells us something true about models: they are simplifications of the real world which enable us to make mathematical predictions about it. RTM is a particular kind of mathematical model designed to merge physical and chemical models of a (bio)geochemical system in order to make either qualitative or quantitative predictions about the system's outputs or processes (Steefel *et al.*, 2005). RTMs merge theoretical calculations of physical and chemical processes with field data from the system being modelled in order to approximate as closely as possible the actual processes in the field. They are highly useful for estimating fluxes in critical natural environments, such as those at the interfaces of contaminants and natural systems. Probably the best-known applications of RTMs are to the impact of contaminant plumes on aquifers and the spread of nuclear waste from storage facilities (Steefel and Van Cappellen, 1998).

By their nature, RTMs are designed to address a specific system or type of system, and no one RTM can encompass all aspects of a particular complex system, especially one as complex as the subsurface interaction at a hydrothermal venting site between

hydrothermal fluid, the rock substratum, and the microbes inhabiting the subsurface environment (Alt-Epping and Diamond, 2008). The simplest kind of RTM is a reaction path model, which steps through equilibrium states via the addition of a reactant to a system. Flow-through models examine the evolution of a fluid's composition as it moves through a 1 or more dimensional space, such as an aquifer. For hydrothermal vent systems, this usually means modelling the 1-D movement of hydrothermal fluid as it is titrated with seawater. Temperature is usually assumed to be a function of dilution rather than modelled based on physical processes. The progress of the reaction is equivalent to travel time along a flowpath (Figure 1.7).



**Figure 0.7: 1-D model of hydrothermal fluid and seawater mixing to produce diffuse venting fluid.**

RTMs have been used to model deep-sea hydrothermal systems since the mid-1970s. Traditionally, deep-sea hydrothermal RTMs have focused on merging the physical and chemical reactions between hydrothermal fluid and rock to predict the mineral precipitation and hydrothermal fluid composition expected at the surface venting site based on the rock type and heat source affecting hydrothermal fluid formation. A number of RTMs have successfully reproduced geological observations based on chemical modelling (Alt-Epping and Diamond, 2008). There have been few attempts to integrate biogeochemical processes into RTMs, largely because there is a lack of data regarding



the *rates* of microbially-mediated reactions which are important in the subseafloor (Geerlings, 2011). Integrating rates into these models requires physiological studies of vent organisms in the laboratory under conditions as close as possible to those in their natural environment. By measuring, in the case of this thesis, rates of methanogenesis under varying conditions in the laboratory, we can apply a reactive transport model to the biogeochemical alteration of hydrothermal fluid in the subseafloor and directly relate CH<sub>4</sub> and H<sub>2</sub> concentrations back to the potential biomass of methanogens in the subseafloor hydrothermal environment. Without direct data on the relationship between biomass production, methane production, and hydrogen consumption, predictions of biomass in these systems are mostly theoretical.

The question then becomes which parameters should be varied when measuring methane production in the laboratory. Hydrogen is, of course, the key variable. It has been demonstrated both that hydrogen determines the distribution of methanogens at our field site (Ver Eecke *et al.*, 2012) and that hydrogen concentrations are a predictor of the major terminal electron acceptor in terrestrial anaerobic environments (Lovley and Goodwin, 1988). In the case of hydrothermal vent systems, carbon dioxide, the electron acceptor, is present in quantities that make it effectively saturated as far as autotrophs are concerned (Ver Eecke *et al.*, 2012). For non-methanogens, electron acceptor concentrations may become relevant; we examine this question in chapter 3 with a sulfur-reducing bacterium. Temperature is well-known as a primary control on biological growth, as demonstrated in the meta-analysis of maintenance energies mentioned above (Tijhuis *et al.*, 1993). This is probably due to basic physiological requirements – as temperature increases, increasing amounts of energy must be expended to maintain membrane permeability (van de

Vossenberg *et al.*, 1995) and to prevent protein denaturation. Hydrothermal vent systems experience significant temperature variation as hydrothermal fluid mixes with seawater, and most thermophilic methanogens are capable of growth over a 30-40°C temperature range, so it is also important to consider how their growth rates and methane production will vary under non-optimal temperatures.

Finally, in autotrophic organisms, measurements of metabolite production translate directly to energy production and usage when compared to growth rates. This means that while maintenance-energy based models are not a focus of this thesis, the data presented can be used to calculate maintenance energies for the organisms studied, based on their minimum hydrogen requirements and changes in methane production over hydrogen concentration and chemostat flow rate, as previously done for other methanogens (Fardeau and Belaich, 1986; Fardeau *et al.*, 1987).

## **1.6 Summary and Research Approach**

This dissertation seeks to explore the use of physiological experiments to constrain growth rates and kinetics of model organisms in the laboratory to make predictions about the distribution and presence of those organisms in extreme environments which are difficult to measure *in situ*. In particular, it seeks to demonstrate how the characterization and growth of organisms from these kinds of environments is crucial to accurately modelling biogeochemical processes, and how this is an effective tool for directing the search for extraterrestrial life.

Specifically, this is done by culturing and isolating representative organisms (*Methanothermococcus* and *Desulfurobacterium*) from hydrothermal vent environments,

particularly low-temperature hydrothermal venting which represents the subsurface environment; examining how the genome of one organisms (*Methanocaldococcus bathoardescens*) helps understand its physiology; measuring the responses of model organisms when grown in the laboratory to key constraints on their growth in the environment, such as temperature and hydrogen availability; collecting culture-dependent and -independent field data on the distribution of these organisms and their growth in microcosm; building a model predicting their abundance in the subsurface based on laboratory measurements of their growth rates and constraints, and comparing it to our field measurements of their substrates, products, and distribution.

It is undeniable that the majority of prokaryotes in the environment are currently unculturable, and that those we can successfully culture in the laboratory represent only a fraction of genetic diversity. However, predictions based on genetic diversity can only go so far. By isolating the organisms we can culture and examining their physiology in the laboratory, under conditions as close as possible to those in their environment, we can measure what they are actually capable of – and this allows us to make accurate predictions of what they may actually be *doing* in environments we cannot sample or perform rate measurement experiments in directly.

## **CHAPTER 2**

# **REACTIVE TRANSPORT MODEL OF METHANOGENESIS IN THE SUBSEAFLOOR OF THE AXIAL VOLCANO HYDROTHERMAL FIELD**

### **2.1 Abstract**

It is estimated that up to a fifth of all biomass on Earth may be contained in the subsurface of the oceanic crust. Estimates of the total amount of subsurface biomass have varied considerably, as it is difficult to sample directly. It is important to quantify the subsurface biomass, as these organisms play crucial roles in biogeochemical cycling, as well as providing an important model environment for potential habitats for life on other planets. Deep-sea hydrothermal vent systems can provide a window into the microbial inhabitants of the subsurface through venting of diffuse hydrothermal fluid. However, the extent of the subsurface biosphere associated with deep-sea hydrothermal vent fields is difficult to assess based directly on diffuse fluid venting, as it is highly diluted with seawater. Hyperthermophilic and thermophilic microbes, particularly hydrogenotrophs, form an important component of subsurface biomass. Here we describe a one-dimensional reactive transport model of hydrogenotrophic methanogenesis in the subsurface of Axial Volcano, based on the kinetics of subsurface methanogens measured in the laboratory, which can be used to provide empirical constraints on their contribution to the subsurface biomass. We have compared this model to the distribution of methanogens at diffuse venting sites at Axial using culture-dependent and -independent field data. Our model uses our laboratory data to predict residence times and cell masses

for methanogens in the subsurface that are consistent with our field data. This type of model – integrating physiological, molecular, and geochemical data – represents a method for constraining subsurface biomass by sampling outflows which is applicable not only to the subsurface biosphere at hydrothermal venting sites, but other environments where it is difficult to make in situ rate measurements.

## **2.2 Introduction**

One of the most under-studied but potentially important biospheres on Earth is the microbial biosphere of the oceanic crust. While estimates of the total potential biomass contained there have varied considerably over the years (Heberling *et al.*, 2010), at their highest suggesting microbial biomass in the subsurface could be greater than that of terrestrial plants (Whitman *et al.*, 1998), the most current estimates suggest that microbial biomass in the oceanic subsurface comprises approximately a fifth of all organic carbon on Earth – a greater percentage than that thought to exist in the terrestrial subsurface and soils combined (Kallmeyer *et al.*, 2012). It is difficult to directly estimate the extent of this biosphere because it is so difficult to sample directly (Edwards *et al.*, 2011). The oceanic subsurface biosphere is important for a number of reasons. It represents a direct interface between geological cycling and recycling of rocks and elements into the Earth's deep crust and mantle and the Earth's biosphere, via autotrophic metabolisms. It also represents an important model environment for potential habitats for life on other planets, as well as a safe haven for life on the early Earth during the Late Heavy Bombardment (Gold, 1992).

Much research into the oceanic subsurface is done via drilling programs (Edwards *et al.*, 2011), but sampling is both difficult and prone to contamination, and it is particularly

hard to measure rates for key microbially-mediated processes. A natural ‘window’ into the subsurface biosphere can be provided by deep-sea hydrothermal vent systems (Orcutt *et al.*, 2011; Deming and Baross, 1993). These represent locations where seawater is entrained into the crust, altered and heated by water/rock reactions in proximity to rising magma, and returned as hot, reducing, metal- and gas-rich hydrothermal fluid. While life probably cannot survive the extreme temperatures of pure hydrothermal fluid, as the known limit for life is around 122°C (Takai *et al.*, 2008), diffuse hydrothermal fluid – which has been diluted with less deeply entrained seawater as it returns to the ocean/crust interface and emerges at temperatures well within the limits of life – carries with it representatives of the subsurface biosphere. Thermophilic and hyperthermophilic organisms, which have optimum growth temperatures above 50°C (Madigan *et al.*, 2012) in diffuse hydrothermal fluid, probably represent the ecology of the subsurface biosphere, as they cannot grow in the cold ocean. By examining the thermophiles and hyperthermophiles in diffuse venting fluid, and their metabolisms and metabolic products, it is possible to model what they might be doing deeper in the subsurface.

Primary production in the hot subsurface biosphere is thought to be driven by hydrogenotrophy, as this is one of the major available electron donors (Nealson *et al.*, 2005; Takai *et al.*, 2004). Major anaerobic hydrogenotrophic metabolisms include the reduction of sulfur compounds and of ferric iron, and methanogenesis. Our field site, Axial Volcano, has been studied for the last twenty-five years (Kelley *et al.*, 2014) and methanogens are an important part of the subsurface community (Huber *et al.*, 2002; Ver Eecke *et al.*, 2012). Hydrogenotrophic methanogenesis is particularly tractable for modelling because it requires only two substrates, one of which (CO<sub>2</sub>) can also be fixed

to create biomass. Formate is a potential alternative electron donor for thermophilic hydrogenotrophic methanogens, and is present at some hydrothermal vent systems, but is probably not significant at Axial (Lang *et al.*, 2010) meaning only hydrogenotrophy needs to be considered as a metabolic mode.

Reactive transport modelling (RTM) is often used to model biogeochemical processes in subsurface terrestrial aquifers and other areas where fluids with different geochemical characteristics are mixing and/or reacting with the surrounding rocks, such as in areas of nuclear waste or oil contamination (Steeffel and Van Cappellen, 1998; Steeffel *et al.*, 2005). In hydrothermal systems it has been used to model water/rock reactions and geochemical changes in hydrothermal fluid (Alt-Epping and Diamond, 2008) as well as fluid moving through hydrothermal vent chimneys (LaRowe *et al.*, 2014; Geerlings, 2011), but to date it has not been used to model the biogeochemical reactions of the hydrothermal vent subsurface biosphere, despite its use in similar situations in terrestrial environments. In this paper, we apply a one-dimensional reactive transport model to hydrogenotrophic methanogenesis in the subsurface of Axial Volcano, our field site. Our model utilizes kinetic data for hydrogenotrophic, thermophilic methanogenesis by organisms native to hydrothermal vent systems measured in continuous flow chemostat culture. Values such as cell yield for reactive transport models are often taken from meta-analyses of cell yield over a wide variety of metabolisms (Roden and Jin, 2011), many of which are very different to the anaerobic, low-energy autotrophy in this sort of system. Our model relies on cell yields and methane production rates for ecologically relevant organisms measured over a range of ecologically relevant temperatures and substrate concentrations. To confirm that methanogens at our field site are not limited by factors

other than temperature and hydrogen availability, we have performed microcosm studies measuring the production of methane by methanogens in diffuse hydrothermal fluid when heated and supplied with additional hydrogen, including whether supplementation with nitrogen raises their growth rates.

Gathering these direct data on methane production and cell yield by our model organisms allows us to model the potential biomass and size of the biosphere for this important mode of primary production in the subsurface at Axial. We have compared this model to the distribution of methanogens at diffuse venting sites at Axial using culture-dependent and independent-field data. Our model predicts a range of subsurface sizes and methanogen populations which are consistent with both our laboratory and our field data. By integrating physiological, molecular, and geochemical data, we can constrain subsurface biomass by sampling outflows at diffuse venting sites. This method is applicable both at other hydrothermal vent sites and for other kinds of metabolisms, if the appropriate physiological measurements are made, but to other environments where it is difficult to make in situ rate measurements.

## **2.3 Methods**

### **2.3.1 Chemostat data collection**

*Methanocaldococcus jannaschii* (MJ) and *Methanothermococcus thermolithotrophicus* (MT) from the DSMZ collection and *Methanothermococcus* strain BW11 (isolated from the High Rise hydrothermal field on the Juan de Fuca Ridge) were grown at 82°C and 65°C (MJ), 65°C and 55°C (MT), and 65°C (BW11) in a 2 L bioreactor with a working volume of 1.5 L. The medium was modified DSMZ 282 medium (Jones *et al.*, 1983; Burggraf *et al.*, 1990) with 1 g L<sup>-1</sup> sodium thiosulfate as a sulfur source, reduced with



0.025%  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ , 0.025% cysteine, and 0.00002%  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ , (herein referred to as 282T). The medium was sparged with  $7.5 \text{ mL min}^{-1}$   $\text{CO}_2$  and varying rates of  $\text{H}_2$  and  $\text{N}_2$  to bring the total gas flow rate to  $70 \text{ mL min}^{-1}$ , or  $100 \text{ mL min}^{-1}$  for higher  $\text{H}_2$  concentrations. It was maintained at pH 6.0 (HYSI 0.1) by the automatic addition of 0.25 mM HCl, and stirred at 300 rpm. For chemostat growth, media was added via peristaltic pump from an 18.5 L reservoir sparged with  $\text{N}_2$  and heated to the same temperature. Organisms were allowed to grow to approximately  $2 \times 10^7 \text{ cells mL}^{-1}$ , then media replaced at various dilution rates until steady-state conditions were reached (assumed to be after 3 full replacements of media.) Cell concentration was determined by cell counts with a Petroff-Hausser counting chamber and phase-contrast light microscope. Headspace was sampled directly with a Hamilton gas-tight syringe through a septum, and 100  $\mu\text{l}$  samples measured for methane concentration on a Shimadzu GC-17A gas chromatograph with flame-ionization detector and a molecular sieve 5A column at  $120^\circ\text{C}$ . Hydrogen was measured on a Shimadzu GC-8A gas chromatograph with a thermal conductivity detector and an Alltech Haysep DB 100/120 column at  $120^\circ\text{C}$ . Gas concentrations in media were measured by anaerobically transferring 20 mL of media into a sealed 60 mL bottle flushed with  $\text{N}_2$ , allowing gases to equilibrate into the headspace, and measuring 100  $\mu\text{l}$  samples of headspace on the two gas chromatographs. Gas flow rates were measured via bubble-meter. Total methane production per cell was calculated by measuring methane concentrations in headspace and medium, and multiplying by the respective rates of change (gas flow and dilution rate in  $\text{mL min}^{-1}$ ) assuming that the total gas flow out was equivalent to the gas flow in. Measurements were taken in duplicate and the standard error calculated.

### 2.3.2 Batch experiments

*M. jannaschii*, *M. thermolithotrophicus*, and *Methanothermococcus* sp. BW11 and FTB11 were grown in 10 ml Balch tubes of 282T medium (see above) and overpressurized with 2 atm H<sub>2</sub>:CO<sub>2</sub> (80:20). For each experiment, 10-12 tubes of media were inoculated simultaneously with 0.1 ml of a culture in logarithmic growth-phase, and incubated in a forced-air oven at the required temperature. Two tubes were removed after approximately two doubling periods and at regular time points thereafter until the cultures reached late logarithmic or early stationary growth phase. After cooling to room temperature, tube headspace was measured with a syringe, 100 µl samples of tube headspace were measured for methane concentration on a Shimadzu GC-17A gas chromatograph with flame-ionization detector and a molecular sieve 5A column at 120°C. Cell concentration was determined by cell counts with a Petroff-Hausser counting chamber and phase-contrast light microscope. Growth rates were calculated with a best-fit exponential curve of cell counts per ml over time, and methane production rates from a best-fit linear curve of total methane production over total cell count per tube (assuming steady rates of methane production per cell doubling). Growth energy was calculated using the following equation:

$$GE \text{ (kJ cell}^{-1} \text{ sec}^{-1}) = \text{CH}_4 \text{ production rate} \times \Delta G_r^\circ \times (\text{doubling time})^{-1}$$

( $\Delta G_r^\circ$  taken from calculated reaction values for methane production in Amend and Shock (2001), and CH<sub>4</sub> production and doubling time from experimental data.)

*Methanothermococcus* strain FTB11 was isolated from the Fuzzy Tubeworm Bush vent at ASHES vent field, Axial Volcano.

### 2.3.3 Most-Probable-Number (MPN) and microcosm experiments

#### 2.3.3.1 MPN experiments

Four types of media were used for MPN enrichments. The methanogen medium was based on DSMZ medium 282 and contained the following (per liter in ddH<sub>2</sub>O): 0.14 g of K<sub>2</sub>HPO<sub>4</sub>, 0.14 g of CaCl<sub>2</sub>·7H<sub>2</sub>O, 0.25 g of NH<sub>4</sub>Cl, 3.4 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.1 g of MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.34 g of KCl, 0.05 mg of NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.05 mg of Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O, 30 g of NaCl, 1 g of NaHCO<sub>3</sub>, 1 g of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.24 g of Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (2012 only), 10 ml of Wolfe's minerals, 10 ml of Wolfe's vitamins, and 50 µl of resazurin. It was pH balanced to 6.0, reduced with 0.0002% (NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub>, 0.025% cysteine and 0.025% Na<sub>2</sub>S·9H<sub>2</sub>O, and pressurized with 2 atm of 80:20 H<sub>2</sub>:CO<sub>2</sub> headspace. There was no growth in any of this media in 2012, presumably due to the added molybdate that was intended to inhibit the growth of sulfate reducers. While *Methanocaldococcus* strain JH146 and *Methanothermococcus* strain FTB11, both isolated from Axial Volcano, grew in the medium with molybdate, the medium may have been too harsh for non-laboratory adapted strains. It was omitted from the medium in 2013. The autotrophic sulfur-reducer medium was the same as the methanogen medium except that 10 g L<sup>-1</sup> of elemental sulfur were added, no molybdate was added, and the medium was reduced with 0.64 mM dithiothreitol (DTT). The heterotroph medium was based on the Adams lab recipe and contained the following (per liter): 0.14 g of K<sub>2</sub>HPO<sub>4</sub>, 0.14 g of CaCl<sub>2</sub>·7H<sub>2</sub>O, 0.25 g of NH<sub>4</sub>Cl, 3.4 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.0 g of MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.34 g of KCl, 2.7 g of Na<sub>2</sub>SO<sub>4</sub>, 18 g of NaCl, 1 g of NaHCO<sub>3</sub>, 0.0002% of (NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub>/(NH<sub>4</sub>)<sub>2</sub>Ni(SO<sub>4</sub>)<sub>2</sub> solution, 10 µM of Na<sub>2</sub>WO<sub>4</sub>/Na<sub>2</sub>SeO<sub>4</sub> solution, 1 g of yeast extract, 5 g of maltose, 10 ml of Adams' minerals, 10 ml of Wolfe's minerals, 50 µl of resazurin, and 10 g of elemental sulfur. It

was pH balanced at 6.8, reduced with 0.025% cysteine-HCl and 0.025%  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ , and pressurized with 2 atm of 80:20  $\text{N}_2:\text{CO}_2$ . Three-tube Most-Probable-Number (MPN) analyses were performed by adding 3.3 ml, 0.33 ml, and 0.03 ml of the hydrothermal fluid samples in triplicate to the three types of media described above in both 2012 and 2013. After inoculation, the tubes were incubated at 55°C or 80°C for up to 7 days onboard ship. Tubes were stored at room temperature during shipping back to our lab on shore. They were only incubated for <7 days if they became visibly turbid during that time. In the lab, growth was confirmed for all four types of media by using phase-contrast light microscopy. Growth of methanogens and  $\text{H}_2$ -producing heterotrophs was determined by analyzing for  $\text{CH}_4$  and  $\text{H}_2$ , respectively, in the headspace using gas chromatography. Growth of sulfur reducers was determined spectrophotometrically by testing for the production of  $\text{HS}^-$  using the methylene blue method. Sulfur-reducer medium was also analyzed for  $\text{CH}_4$ , as the addition of elemental sulfur does not suppress methanogen growth.

#### 2.3.3.2 Microcosm incubations

Hydrothermal fluid (25 ml) was added to a sealed 60 ml serum bottle flushed with either  $\text{H}_2:\text{CO}_2$  (high hydrogen and high hydrogen/high ammonia analyses) or  $\text{N}_2:\text{CO}_2$  (low hydrogen and no hydrogen analyses). 1 ml of  $\text{H}_2:\text{CO}_2$  was added to the  $\text{N}_2:\text{CO}_2$  bottles to produce a concentration of approximately 20  $\mu\text{M}$  hydrogen in the fluid sample (discounting any present originally). In 2012, 4.7 mM  $\text{NH}_4\text{Cl}$  were added to the high hydrogen/high ammonia bottles (the same as our standard media). In 2013, the ammonium concentration was reduced to 47  $\mu\text{M}$   $\text{NH}_4\text{Cl}$  due to concerns about possible growth inhibition (based on 2012 results that in hindsight were likely due to low cell

concentrations). Hydrothermal fluid samples were taken from the same sample bags for all incubation sites except Marker 113, where they were taken from a second fluid sample from the same vent. Duplicates of each condition (high H<sub>2</sub>, high H<sub>2</sub>/high NH<sub>4</sub><sup>+</sup>, low H<sub>2</sub>, no H<sub>2</sub>) were incubated at 55°C or 80°C for up to 4 weeks. Growth of methanogens was determined by analyzing for CH<sub>4</sub> in the headspace using gas chromatography.

### 2.3.4 Metagenome and metatranscriptome analysis

#### 2.3.4.1 Sampling and extraction

Five filter holders charged with RNeasy lysis buffer and containing a 0.2 µm pore size, 47 mm diameter flat filter were loaded onto the Hydrothermal Fluid and Particle Sampler (HFPS). At each vent site, 3 L was pumped through each filter and flooded with RNeasy lysis buffer. Once on deck, filters were removed from their holders, folded into quarters and placed into sterile 50 mL tubes with ~15 mL of RNeasy lysis buffer. Tubes were kept at 4°C for 24 hours and then moved to -80°C. The filters were first cut in half with a sterile razor, with half used for DNA and half used for RNA extraction. RNA was extracted using the mirVana miRNA isolation kit (Ambion) with an added bead-beating step using RNeasy PowerSoil beads (MoBio). A total volume of 100 µl was extracted and was then DNase treated using the Turbo-DNase kit (Ambion), purified, and concentrated using the RNeasy MinElute kit (Qiagen). Ribosomal RNA removal, cDNA synthesis, and metatranscriptomic library preparation was carried out using the Ovation Complete Prokaryotic RNA-Seq DR multiplex system (Nugen) following manufacturer's instructions. Prior to library construction, cDNA was sheared to a fragment size of 175 bp using a Covaris S-series sonicator. For DNA extraction, the DNA filter was first rinsed

with sterile PBS to remove RNAlater and then was extracted using a phenol-chloroform method adapted from Crump et al. (Crump *et al.*, 2003) and Zhou et al. (Zhou *et al.*, 1996). DNA was then sheared to a fragment size of 175 bp using a Covaris S-series sonicator. Metagenomic library construction was completed using the Ovation Ultralow Library DR multiplex system (Nugen) following manufacturer's instructions. Metagenomic and metatranscriptomic sequencing was performed on an Illumina HiSeq 1000 at the W.M. Keck sequencing facility at the Marine Biological Laboratory. All libraries were paired-end, with a 30 bp overlap, resulting in an average merged read length of 160 bp.

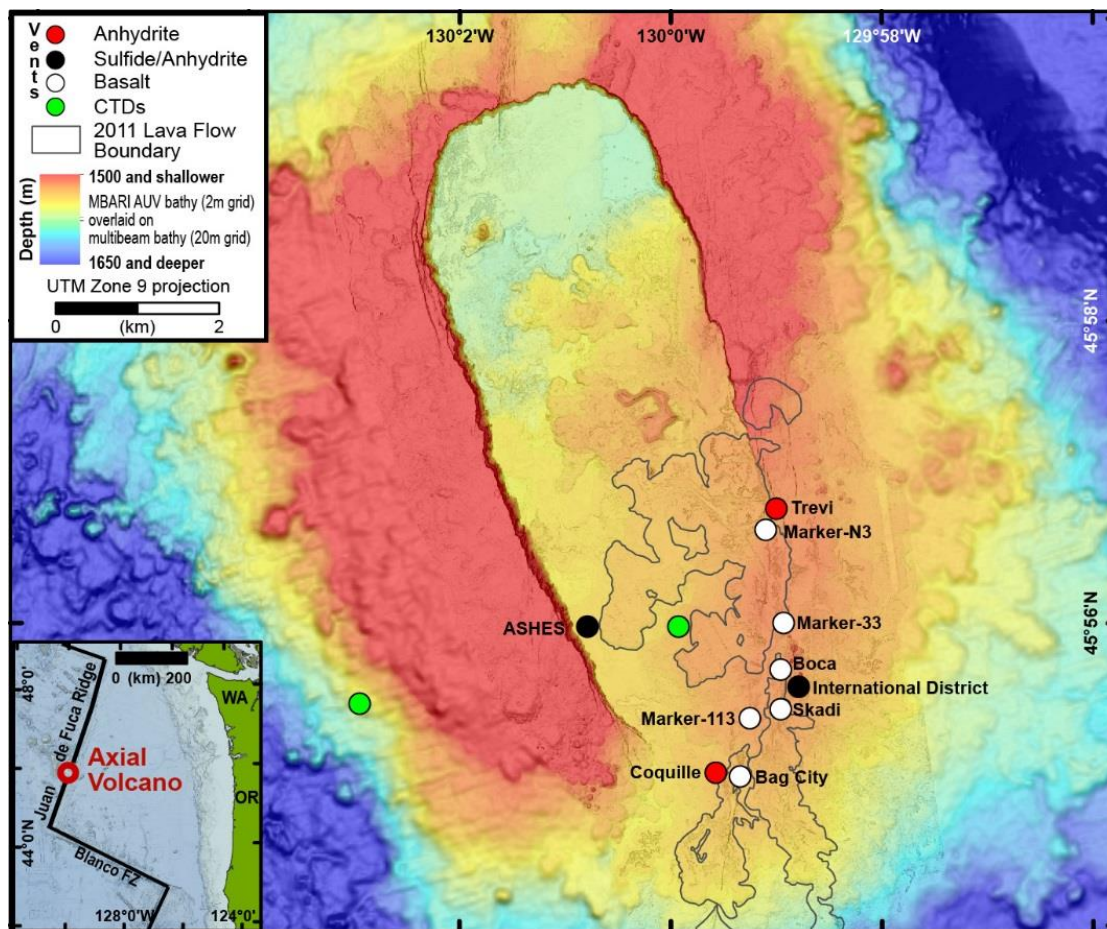
#### 2.3.4.2 Library analyses

For all metagenomic and metatranscriptomic libraries, paired-end partially overlapping reads were merged and quality filtered using custom Illumina utility scripts (<https://github.com/meren/illumina-utils>). Merged reads were dereplicated then assembled using CLC Genomics Workbench (v 7.0) using default settings and a minimum contig length of 200 bp. Dereplicated libraries were only used for easing assembly, mapping was completed using all reads. Assembled contigs from each library were submitted to the DOE Joint Genome Institute's Integrated Microbial Genome Metagenomic Expert Review (IMG/MER) annotation pipeline for Open Reading Frame (ORF) identification and functional and taxonomic annotation (Markowitz *et al.*, 2012). To determine the number of reads per annotated ORF, reads from each library were mapped to ORFs using CLC Genomics Workbench (v 7.0), using default settings (50% percent identity and 80% minimum length fraction). To identify rRNA reads in the metatranscriptomes, reads were mapped to SILVA SSU and LSU databases release 111

(Pruesse *et al.*, 2007) using Bowtie2 v. 2.0.0-beta5 (Langmead and Salzberg, 2012) with a local alignment and default settings. Identified rRNA reads were separated from each metatranscriptome using custom Perl scripts. Ribosomal RNA from metagenomes was also identified using this method but reads were not separated. Once rRNA was identified, 16S rRNA reads were specifically identified by mapping rRNA reads to the Greengenes 16S rRNA taxonomic database, May 2013 release (McDonald *et al.*, 2012) using Bowtie2. 16S rRNA reads were taxonomically identified with MOTHUR v. 1.33 (Schloss *et al.*, 2009) using the Greengenes taxonomic database. ORFs from each library were annotated against the KEGG ontology (KO) database. Only annotations with minimum requirements of an e-score of  $1e^{-10}$ , 30% amino acid identity, and alignment length of 40 amino acids were included in functional analyses. KO abundances for each metagenome were normalized by dividing each KO annotation by the number of hits to DNA-directed RNA polymerase, beta subunit gene (*rpoB*). Metatranscriptomes were normalized using the following ratio: ((number of hits to each KO/total annotated transcripts)/(number of hits to *rpoB* in the metagenome/total annotated metagenomic reads)).

## 2.4 Results and Discussion

### 2.4.1 Geochemistry and microbiology at Axial Volcano



**Figure 2.1:** Site map of Axial Volcano showing hydrothermal vent locations and sampling sites. The green circles show the locations of background seawater hydrocasts. The inset shows the location of Axial Volcano in the northeastern Pacific Ocean.

Our field site, Axial Volcano, is located on the Juan de Fuca mid-ocean ridge spreading center in the northeastern Pacific, approximately 1,500 m below sea level (Johnson and Embley, 1990). It is an active volcano with lava flow eruptions observed in 1998 and 2011 (Chadwick *et al.*, 2012; Clague *et al.*, 2013) and hosts both diffuse and sulfidic hydrothermal venting at the southern end of its caldera (Figure 1). The eastern side of the caldera has basaltic- and sulfidic-hosted vents, and the western venting site (ASHES) has



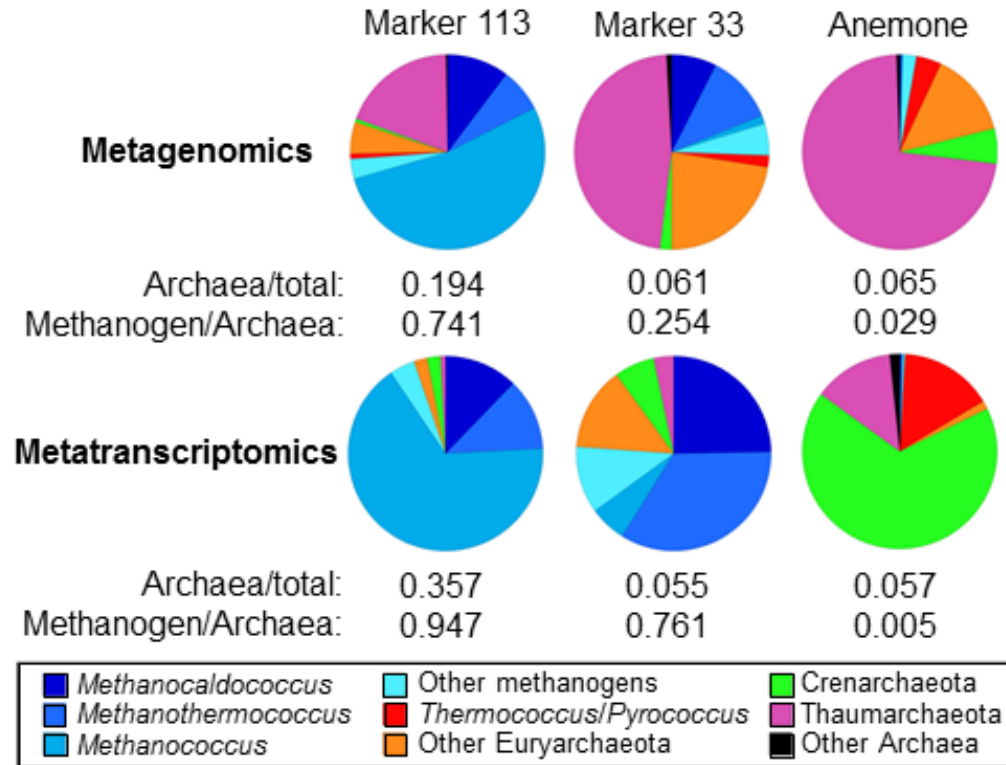
primarily anhydrite-sulfide venting. Fluid chemistry at these vents has been monitored since 1986 (Butterfield *et al.*, 2004), and microbiology since 1998 (Huber *et al.*, 2002, 2003). In 2013, we collected diffuse hydrothermal fluid samples from seven vents, one at ASHES and six in the eastern caldera venting fields, using the Hydrothermal Fluid and Particle Sampler (Edmond *et al.*, 1992; Butterfield *et al.*, 1997) attached to ROV Jason II and ROV ROPOS. Hydrogen and methane concentrations varied significantly between these vent sites (Table 1).

**Table 2.1: Axial Volcano diffuse hydrothermal fluid characteristics**

	Marker N3	Marker 33	Boca	Skadi	Marker 113	ASHES	International District
<b>Fluid</b>							
<b>characteristics:</b>							
Temperature	19°C	27°C	7°C	35°C	24°C	29°C	24°C
pH (room T)	5.0	5.5	6.9	6.2	6.2	5.5	5.4
H <sub>2</sub> (μmol kg <sup>-1</sup> )	< 1	1.3	< 1	< 1	< 1	10	< 1
CH <sub>4</sub> (μmol kg <sup>-1</sup> )	67	30	< 1	5	18	15	3
ΣNH <sub>3</sub> (μmol kg <sup>-1</sup> )	1.4	3.5	2.3	7.4	6.4	2.7	3.3
ΣH <sub>2</sub> S (μmol kg <sup>-1</sup> )	539	533	4	83	626	1,077	255

We measured methanogen distribution by examining the percentage of transcripts which clustered to known methanogens in metagenome and metatranscriptome sequences from three specific venting sites (Figure 2). We also performed Most-Probable-Number (MPN) assessments of hyperthermophilic and thermophilic methanogen distribution (Table 2). We found that hyperthermophilic and thermophilic methanogens were present in both culture-dependent and culture-independent measurements at all our vent sites, but their relative importance varied significantly. At some vents, notably Marker 113 and Marker 33 on the eastern side of the caldera, methanogens represented most of the archaeal metagenomic and metatranscriptomic sequences; in particular, at Marker 113 methanogenic sequences comprised over a third of the entire metatranscriptome, consistent with high numbers measured in MPN analyses and the significant CH<sub>4</sub> anomaly in Marker 113 fluid. However, at Anemone, on the western side of the caldera, despite the presence of some methanogens at thermophilic temperatures in MPN analyses, methanogen sequences were essentially absent from both the metagenome and metatranscriptome and present in very low numbers ( $<10^3$  cells L<sup>-1</sup>) in the MPNs. The MPN experiments also showed that hyperthermophilic and thermophilic heterotrophs were present at every vent site studied (in at least one of the two years), demonstrating the diffuse fluid did represent organisms from the hot subsurface biosphere. At most but not all sites where methanogens were present, hydrogenotrophic sulfidogens were also present in similar or smaller numbers – the major exception being the Anemone vent, where hyperthermophilic and thermophilic sulfidogens outnumbered methanogens (Table 2).

**Figure 2.2: Metagenomics and metatranscriptomics of selected vents at Axial Volcano**



Methanogens at our field site, Axial Volcano on the Juan de Fuca Ridge, are overwhelmingly dominated by one family, the *Methanococcales* (Figure 2). Three of the four genera of the *Methanococcales*, *Methanothermococcus*, *Methanotorris*, and the *Methanocaldococcus*, are thermophilic or hyperthermophilic and autotrophic. Based on our experiments, the *Methanothermococci* and *Methanocaldococci* appear to be constrained only by temperature and availability of hydrogen, their sole electron donor. Based on culture-dependent and independent-sampling at our field site, Axial Volcano, these two genera are an important part of the anaerobic, autotrophic community in the subsurface at this site (*Methanotorris* sequences are rare). While mesophilic methanogen sequences (the *Methanococci*) do comprise significant portions of methanogen sequences at both Marker 113 and Marker 33, these sequences almost all group with *Methanococcus aeolicus* (data not shown). *M. aeolicus* is moderately thermophilic and has a maximum

temperature significantly higher than the other *Methanococci* (Whitman and Jeanthon, 2006). We therefore predict that the majority of methanogenesis at Axial is taking place in the thermophilic subsurface, where the fluid is still anoxic. At Anemone, where methanogens are essentially absent in ‘omics sequences and in very low numbers in culture-dependent MPN experiments, sulfidogenic hydrogenotrophs – reducing thiosulfate and/or sulfur – are found in both MPNs and ‘omics sequences. The sequences are primarily from known hydrogenotrophic sulfur- and thiosulfate-reducers in the phylum *Aquificales* (data not shown).

We assume that the abundance of methanogens as indicated by the metagenomic/metatranscriptomic sequences and total cell counts in our diffuse fluid samples represents an upper bound on methanogen concentration in the fluid, whereas our abundance estimates from MPN analyses indicate a lower bound. The remaining question is what controls the distribution of methanogens across these different vent/subsurface sites.

**Table 2.2: Most-Probable-Number (MPN) estimates for the 2012-2013 Axial Volcano Cruises at 80°C. Cell concentrations are in cells L<sup>-1</sup>, with the three-tube MPN scores in brackets (\*microcosm run, \*\*microcosm growth).**

Sample	Adams medium + S°			282 medium		282 medium + S°			Total cells
	cells	H <sub>2</sub>	CH <sub>4</sub>	Cells	CH <sub>4</sub>	cells	S <sup>2-</sup>	CH <sub>4</sub>	
<b>80°C incubations:</b>									
J660-19 (Anemone)*	7,200 (3-3-0)	2,790 (3-2-0)	ND (0-0-0)			270 (2-0-0)	ND (0-0-0)	ND (0-0-0)	7.9 × 10 <sup>7</sup>
J660-23 (Fuzzy TWB)*	2,790 (3-2-0)	2,790 (3-2-0)	ND (0-0-0)			1,290 (3-1-0)	ND (0-0-0)	ND (0-0-0)	4.7 × 10 <sup>7</sup>
J661-19 (Boca)	690 (3-0-0)	ND (0-0-0)	ND (0-0-0)			270 (2-0-0)	ND (0-0-0)	ND (0-0-0)	2.3 × 10 <sup>8</sup>
J661-21 (Marker 113)*	2,790 (3-2-0)	210 (1-1-0)	ND (0-0-0)			210 (1-1-0)	ND (0-0-0)	120 (1-0-0)	3.4 × 10 <sup>8</sup>
J726-21 (Anemone)**	13,800 (3-3-1)	1,290 (3-1-0)	270 (2-0-0)	690 (3-0-0)	270 (2-0-0)	1,290 (3-1-0)	1,290 (3-1-0)	120 (1-0-0)	4.1 × 10 <sup>8</sup>
J726-19 (Vixen)	6,300 (3-2-3)	ND (0-0-0)	ND (0-0-0)	ND (0-0-0)	ND (0-0-0)	1,050 (2-2-3)	210 (1-1-0)	210 (1-1-0)	1.4 × 10 <sup>8</sup>
J728-21 (El Guapo)**	>33,000 (3-3-3)	120 (1-0-0)	ND (0-0-0)	ND (0-0-0)	ND (0-0-0)	210 (1-1-0)	120 (1-0-0)	ND (0-0-0)	6.8 × 10 <sup>7</sup>
J730-19 (Marker N3)*	>33,000 (3-3-3)	ND (0-0-0)	ND (0-0-0)	ND (0-0-0)	ND (0-0-0)	120 (1-0-0)	ND (0-0-0)	ND (0-0-0)	4.1 × 10 <sup>8</sup>
R1663-21 (Marker 113)**	>33,000 (3-3-3)	330 (1-1-2)	120 (1-0-0)	1,050 (2-3-2)	1,050 (2-3-2)	6,300 (3-2-2)	90 (0-1-0)	1,080 (2-3-1)	5.4 × 10 <sup>8</sup>
R1663-19 (Skadi)**	3,600 (3-1-2)	330 (1-2-0)	ND (0-0-0)	>33,000 (3-3-3)	13,800 (3-3-1)	1,290 (3-1-0)	210 (1-1-0)	210 (1-1-0)	5.6 × 10 <sup>8</sup>
R1665-19 (Marker 33)**	>33,000 (3-3-3)	7,200 (3-3-0)	ND (0-0-0)	13,800 (3-3-1)	13,800 (3-3-1)	120 (1-0-0)	ND (0-0-0)	120 (1-0-0)	2.8 × 10 <sup>8</sup>
R1665-21 (Boca)**	>33,000 (3-3-3)	ND (0-0-0)	ND (0-0-0)	690 (3-0-0)	276 (2-0-0)	120 (1-0-0)	120 (1-0-0)	ND (0-0-0)	5.0 × 10 <sup>8</sup>
Off-summit CTD*	ND (0-0-0)	-	-			ND (0-0-0)	-	-	2.5 × 10 <sup>7</sup>

Low NH<sub>4</sub><sup>+</sup> 282 Medium:

R1663-21 (Marker 113): cells = 3-3-2 CH<sub>4</sub> = 3-3-2

R1665-19 (Marker 33): cells = 3-1-1 CH<sub>4</sub> = 3-0-0

**Table 2.3: Most-Probable-Number (MPN) estimates for the 2012-2013 Axial Volcano Cruises at 55°C. Cell concentrations are in cells L<sup>-1</sup>, with the three-tube MPN scores in brackets (\*microcosm run, \*\*microcosm growth).**

Sample	Adams medium + S <sup>o</sup>			282 medium		282 medium + S <sup>o</sup>			Total cells
	cells	H <sub>2</sub>	CH <sub>4</sub>	cells	CH <sub>4</sub>	cells	S <sup>2-</sup>	CH <sub>4</sub>	
<b>55°C incubations:</b>									
J660-19 (Anemone)*	690 (3-0-0)	ND (0-0-0)	ND (0-0-0)			270 (2-0-0)	ND (0-0-0)	ND (0-0-0)	7.9 × 10 <sup>7</sup>
J660-23 (Fuzzy TWB)*	270 (2-0-0)	270 (2-0-0)	ND (0-0-0)			6,300 (3-2-2)	270 (2-0-0)	ND (0-0-0)	4.7 × 10 <sup>7</sup>
J661-19 (Boca)	270 (2-0-0)	ND (0-0-0)	ND (0-0-0)			450 (2-1-0)	90 (0-1-0)	120 (1-0-0)	2.3 × 10 <sup>8</sup>
J661-21 (Marker 113)**	ND (0-0-0)	-	-			270 (2-0-0)	ND (0-0-0)	ND (0-0-0)	3.4 × 10 <sup>8</sup>
J726-21 (Anemone)**	7,200 (3-3-0)	ND (0-0-0)	ND (0-0-0)	690 (3-0-0)	690 (3-0-0)	2,790 (3-2-0)	210 (1-1-0)	120 (1-0-0)	4.1 × 10 <sup>8</sup>
J726-19 (Vixen)	6,300 (3-2-3)	ND (0-0-0)	ND (0-0-0)	7,200 (3-3-0)	690 (3-0-0)	6,300 (3-2-2)	450 (2-1-0)	ND (0-0-0)	1.4 × 10 <sup>8</sup>
J728-21 (El Guapo)*	>33,000 (3-3-3)	ND (0-0-0)	ND (0-0-0)	ND (0-0-0)	ND (0-0-0)	4,500 (3-2-1)	690 (3-0-0)	ND (0-0-0)	6.8 × 10 <sup>7</sup>
J730-19 (Marker N3)*	>33,000 (3-3-3)	ND (0-0-0)	ND (0-0-0)	120 (1-0-0)	120 (1-0-0)	840 (2-2-1)	ND (0-0-0)	120 (1-0-0)	4.1 × 10 <sup>8</sup>
R1663-21 (Marker 113)**	>33,000 (3-3-3)	120 (1-0-0)	120 (1-0-0)	450 (1-2-3)	330 (1-1-2)	6,300 (3-2-2)	90 (0-1-0)	690 (3-0-0)	5.4 × 10 <sup>8</sup>
R1663-19 (Skadi)**	>33,000 (3-3-3)	ND (0-0-0)	ND (0-0-0)	1,290 (3-1-0)	450 (2-1-0)	690 (3-0-0)	270 (2-0-0)	ND (0-0-0)	5.6 × 10 <sup>8</sup>
R1665-19 (Marker 33)**	>33,000 (3-3-3)	ND (0-0-0)	ND (0-0-0)	330 (1-1-2)	330 (1-1-1)	33,000 (3-3-2)	X (1-2-1)	690 (3-0-0)	2.8 × 10 <sup>8</sup>
R1665-21 (Boca)**	>33,000 (3-3-3)	270 (2-0-0)	120 (1-0-0)	690 (3-0-0)	270 (2-0-0)	630 (2-2-0)	450 (2-1-0)	ND (0-0-0)	5.0 × 10 <sup>8</sup>
Off-summit CTD*	ND (0-0-0)	-	-			ND (0-0-0)	-	-	2.5 × 10 <sup>7</sup>

Low NH<sub>4</sub><sup>+</sup> 282 Medium:

R1663-21 (Marker 113): cells = 3-3-3 CH<sub>4</sub> = 3-3-3

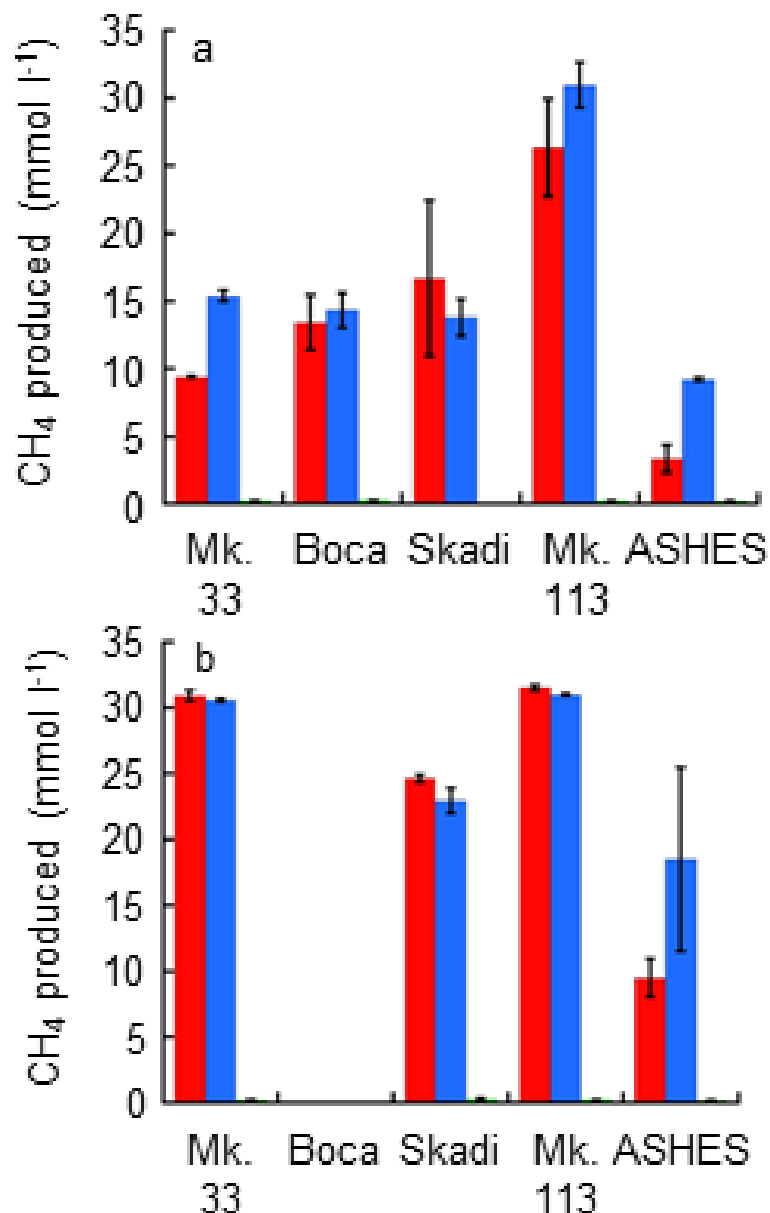
R1665-19 (Marker 33): cells = 1-3-2 CH<sub>4</sub> = 1-3-2

**Table 2.4: End-point methane concentrations (total  $\mu$ moles in headspace) following incubation of hydrothermal fluid with varying amounts of  $H_2$  in the headspace with and without the addition of 4.7 mM  $NH_4Cl$ . ND, no peak(s) detected**

**\*The 2012 80°C incubations for Marker 113 were in duplicate, while the 55°C incubations for Marker 113 were singles due to low sample volume. All other incubations at both temperatures were in duplicate.**

Sample	55°C				80°C			
	High $H_2$	High $H_2$ + $NH_4^+$	Low $H_2$	No $H_2$	High $H_2$	High $H_2$ + $NH_4^+$	Low $H_2$	No $H_2$
Anemone (J726-21)	83.6 $\pm$ 25.2	230.5 $\pm$ 3.4	4.9 $\pm$ 0.9	ND	236.8 $\pm$ 35.8	462.6 $\pm$ 175.0	4.8 $\pm$ 0.4	ND
Anemone (J660-19)	ND	ND	ND	ND	ND	ND	ND	ND
Marker 113 (R1663-21)	658.5 $\pm$ 90.3	774.2 $\pm$ 42.1	6.0 $\pm$ 0.3	ND	788.7 $\pm$ 5.6	775.2 $\pm$ 1.9	6.3 $\pm$ 0.1	ND
Marker 113* (J661-21)	47.3	ND	3.5	ND	ND	ND	ND	ND
Skadi (R1663-19)	417.0 $\pm$ 144.3	344.7 $\pm$ 34.4	ND	ND	616.1 $\pm$ 7.2	573.5 $\pm$ 23.6	6.8 $\pm$ 2.2	ND
Marker 33 (R1665-19)	235.3 $\pm$ 1.5	384.6 $\pm$ 9.8	6.4 $\pm$ 0.1	ND	773.0 $\pm$ 10.0	764.3 $\pm$ 3.0	5.7 $\pm$ 0.2	ND
Boca (R1665-21)	335.3 $\pm$ 50.9	357.5 $\pm$ 32.6	6.7 $\pm$ 0.1	ND	508.1	ND	ND	ND
El Guapo (J728-21)	ND	ND	ND	ND	194.9	601.4	ND	ND
Marker N3 (J730-19)	ND	ND	ND	ND	ND	ND	ND	ND
Fuzzy TWB (J660-23)	ND	ND	ND	ND	ND	ND	ND	ND
Off-summit CTD	ND	ND	ND	ND	ND	ND	ND	ND

## 2.4.2 Methane production experiments



**Figure 2.3: Microcosm experiments. A and B: methane production by microcosm cultures from Axial Volcano diffuse hydrothermal venting at 55°C (a) and 80°C (b); high H<sub>2</sub> is red, high H<sub>2</sub> + NH<sub>4</sub>Cl is blue, low H<sub>2</sub> is green/small bars.**

To assess the nature of in-situ controls on methanogens, we conducted microcosm experiments at 55°C and 80°C (thermophilic and hyperthermophilic temperatures) using diffuse hydrothermal fluid supplemented by differing levels of H<sub>2</sub> and CO<sub>2</sub> and/or amended with NH<sub>4</sub>Cl, to test for nitrogen limitation (Table 3, Figures 3a and 3b).



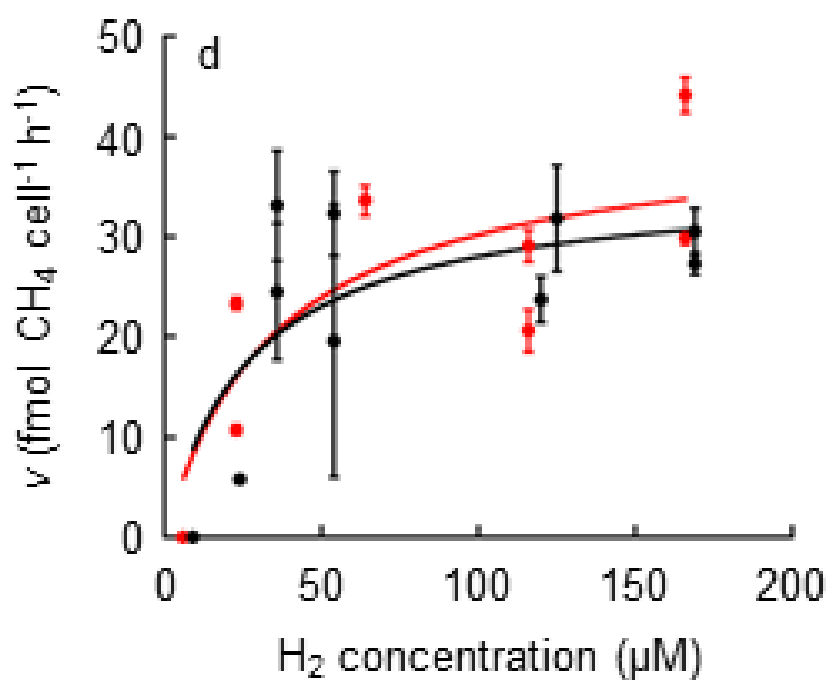
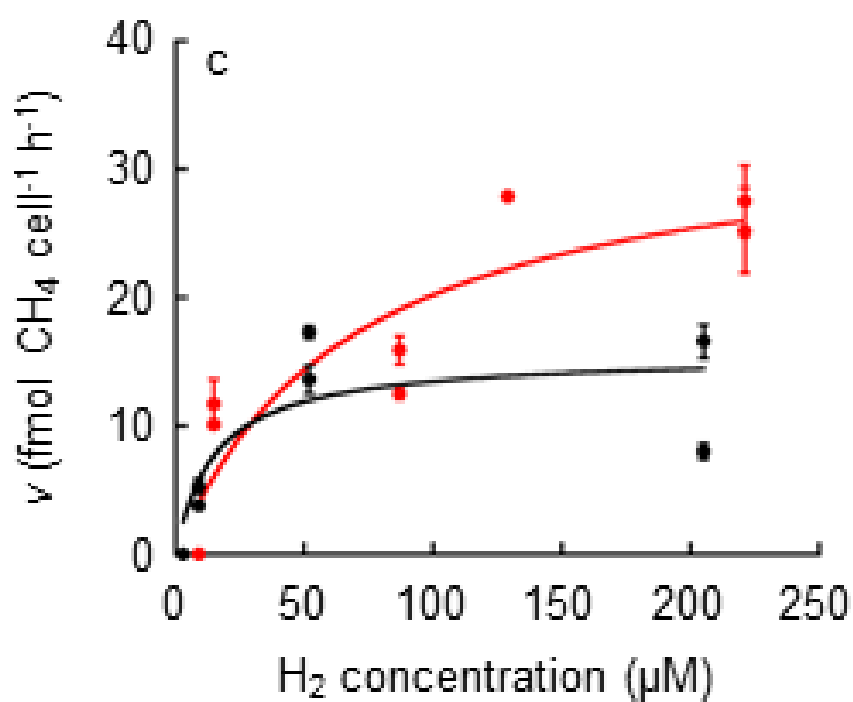
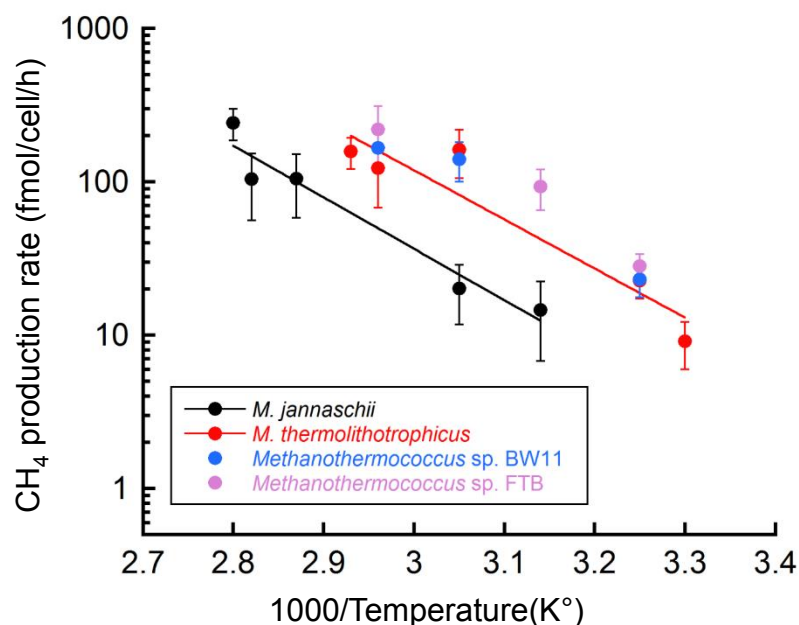


Figure 2.4: Chemostat experiments. C: methane production per cell in continuous flow chemostat culture by *Methanocaldococcus jannaschii* (82°C, red and 65°C, black). D: methane production per cell in continuous flow chemostat culture by *Methanothermococcus thermolithotrophicus* (65°C, red and 55°C, black).



**Figure 2.5: Arrhenius kinetics of thermophilic and hyperthermophilic methanogens grown in Balch tubes.**

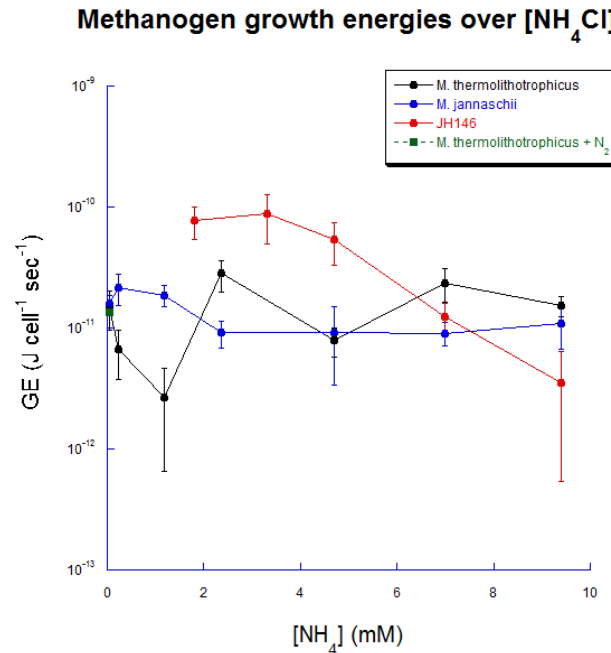
*M. jannaschii*:  $A = 4.41 \times 10^{-4}$  mol CH<sub>4</sub>/cell/h,  $E_a = 64.33$  kJ/mol.

*M. thermolithotrophicus*:  $A = 4.91 \times 10^{-4}$  mol CH<sub>4</sub>/cell/h,  $E_a = 61.36$  kJ/mol.

We saw significant CH<sub>4</sub> production when diffuse fluid was incubated with an excess of H<sub>2</sub>/CO<sub>2</sub>; trace production with a previously-measured (Ver Eecke *et al.*, 2012) minimum H<sub>2</sub> requirement; and no methane production when we did not provide H<sub>2</sub> (beyond background levels in the fluid, which were below detection at all but one site, Marker 113). This indicated that methanogens at Axial were capable of growth with only the addition of H<sub>2</sub>:CO<sub>2</sub>.

The addition of NH<sub>4</sub>Cl did not consistently elevate total CH<sub>4</sub> production. We investigated this because our standard laboratory medium contains 4.7 mM NH<sub>4</sub>Cl, significantly above measured environmental levels at Axial diffuse vents, which are in the micromolar range (Table 1). Experiments with *Methanocaldococcus bathoardescens*, isolated from Axial Volcano (Ver Eecke, 2011; Stewart *et al.*, 2015) showed that its methane

production and growth energy fell when it was grown on media containing more than this concentration of nitrogen. However, the same batch culture experiments performed with *Methanocaldococcus jannaschii* ( $T_{\text{opt}}$  82°C) and *Methanothermococcus thermolithotrophicus* ( $T_{\text{opt}}$  65°C), showed no increase in methane production and/or growth energy when bioavailable nitrogen levels were decreased to near-environmental levels, or drop at levels higher than 4.7 mM. Our microcosm experiments are consistent with these results, and indicate that the *Methanothermococci* and *Methanocaldococci* are not restricted at Axial by nitrogen limitation (except perhaps at Anemone vent, where methanogens are a minor component of the overall microbial population). Furthermore, both *M. jannaschii* and *M. bathoardescens* grow at their optimum rates on laboratory media (282T) when Wolfe's vitamins are removed from the medium, indicating that they are fully autotrophic and do not require any organic supplementation (data not shown).



**Figure 2.6: Growth energies of *M. jannaschii*, *M. bathoardescens*, and *M. thermolithotrophicus* at different concentrations of  $\text{NH}_4\text{Cl}$  (and with additional  $\text{N}_2$  at lowest  $\text{NH}_4$  concentration for *M. thermolithotrophicus*). *M. bathoardescens* data is from Ver Eecke et al. (2013).**

Therefore, when we analyzed the kinetics of methanogenesis in the laboratory, we looked at how methane production by model thermophilic and hyperthermophilic methanogens changed over temperature and H<sub>2</sub> concentration, as these appeared to be the two most important variables in the environment that affecting methanogen growth. Firstly, we measured the Arrhenius constants for methanogenesis for *Methanocaldococcus jannaschii*, *Methanothermococcus thermolithotrophicus*, and *Methanothermococcus* sp. BW11 and FTB11 (isolated from the Endeavour Segment and the ASHES vent field at Axial Seamount, respectively). They were grown over their entire respective temperature ranges, with H<sub>2</sub> concentrations kept the same (~1.2 mM). Results are shown in Figure 2.5. Previous experiments with *Methanocaldococcus bathoardescens* (Ver Eecke *et al.*, 2013) had suggested that the growth energy requirements of hyperthermophilic methanogens remained constant over most of their growth range, only increasing significantly at temperatures very close to the upper limit of growth. However, our experiments showed methane production (and therefore growth energies) increased gradually with temperature for all the organisms tested. Additionally, a previous meta-analysis of maintenance energies (Tijhuis *et al.*, 1993) showed a correlation between optimum growth temperature and maintenance energy. We observed that the range of growth energies was the same for *Methanocaldococcus* and *Methanothermococcus* species, despite the ~20°C difference in optimum temperature, i.e. the Arrhenius constants were very similar. What differed was the temperature *range*, i.e. the activation energy. This demonstrates the necessity of measuring maintenance energy requirements over the full range of temperatures an organism may grow at in the environment, rather than just their temperature optima. It also suggests that the rate of methanogenesis for

these organisms is ultimately constrained by their metabolism – which performs the same processes using the same enzymes - rather than by their specific optimum temperature.

Secondly, we measured the Monod constants for methanogenesis for

*Methanocaldococcus jannaschii*, *Methanothermococcus thermolithotrophicus*, and *Methanothermococcus* sp. BW11. Results are shown in Table 2.5 and Figures 2.3c and 2.3d.

**Table 2.5: Monod constants ( $k_s$  and  $V_{max}$ ) for *M. jannaschii*, *M. thermolithotrophicus*, and *Methanothermococcus* sp. BW11.**

*Methanocaldococcus jannaschii:*

Temp.	$k_s$ ( $\mu\text{M}$ )	$V_{max}$ (fmol/cell/h)	R
82°C	46.9	43.7	0.83
65°C	18.4	33.9	0.75

*Methanothermococcus spp:*

	Temp.	$k_s$ ( $\mu\text{M}$ )	$V_{max}$ (fmol/cell/h)	R
Mthe	65°C	68.5	34.1	0.88
Mthe	55°C	15.2	15.5	0.82
BW11	65°C	65.7	43.4	0.96

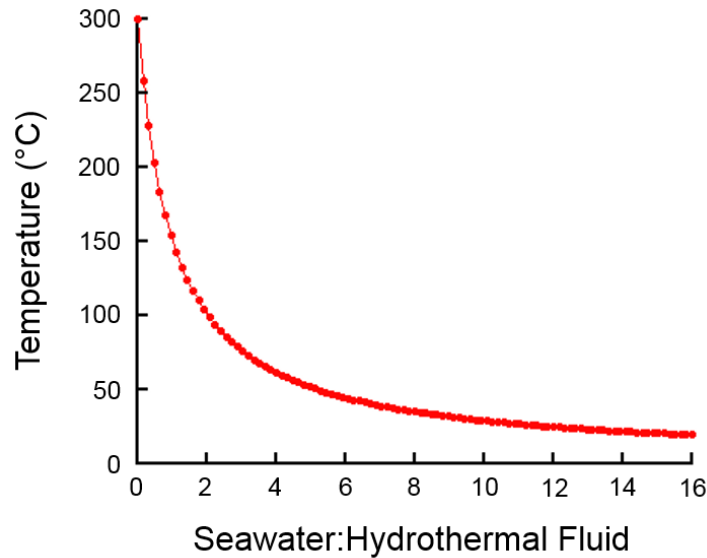
Growth over a range of H<sub>2</sub> concentrations, at two temperatures for both *M. jannaschii* and *M. thermolithotrophicus*, showed that minimum H<sub>2</sub> requirements for these organisms are temperature-dependent and “hyperthermophilic” and “thermophilic” methanogens may in fact compete for hydrogen in the overlapping portion of their temperature ranges, meaning that subsurface populations will be composed of some fraction of both groups for most temperatures below 80°C (Figures 3c and 3d). Minimum hydrogen requirements were lower than previously measured (Ver Eecke *et al.*, 2012), as low as ~9 µM H<sub>2</sub> (for *Methanothermococcus* sp. and *M. jannaschii* when grown at 65°C). Again, this demonstrates that organisms’ energetic fitness in an environment cannot be examined solely by their growth at their optimum temperature.

#### 2.4.3 Reactive transport modelling in the hydrothermal vent subsurface

It is possible to model growth in the subsurface given knowledge of the chemical composition of the hydrothermal endmember fluids and the seawater they are diluted by to form diffuse hydrothermal fluid. Notable examples are the models proposed by McCollom and Shock (1997) and Smith and Shock (2007), which use geochemical data to predict which metabolisms should be favored at different temperatures in the vent systems modelled. However, these models do not use measured constraints for growth rates and metabolite production for the metabolisms they model; rather, a threshold for growth potential based on the energy required to produce a molecule of ATP is used (Hoehler, 2004) or thresholds based on meta-analyses of cell yield over a wide variety of metabolisms (Roden and Jin, 2011). Hydrogenotrophic methanogenesis is an almost ideal process for modelling potential microbial activity in the subsurface, due to the fact that it has no alternate pathways and consists of easily-measured substrates and products - H<sub>2</sub>,

CO<sub>2</sub>, and CH<sub>4</sub> (Ferry, 2010). Our model uses the measured kinetics of methane production by vent methanogens to predict the cell concentrations and residence time necessary to produce the observed methane anomalies at three vent sites: Marker 113 (abundant methanogens), Marker 33 (some methanogens), and Anemone (few methanogens.)

The model is a one-dimensional reactive transport flow model, which can be thought of as a series of boxes, each box representing a different stage in the mixing profile of hydrothermal fluid and seawater. Source hydrothermal fluid (assumed to be represented by the nearest high-temperature venting site to the diffuse fluid vent modelled) is diluted with seawater in step-wise fashion until the temperature and fluid composition match that of the diffuse fluid vented.



**Figure 2.7: Temperature of mixed fluid vs. proportion of seawater to hydrothermal fluid. Growth of methanogens is only possible where the temperature is below 120°C. Temperature is assumed to be a conservative tracer of mixing between seawater and hydrothermal fluid in this model.**

For each model step, methanogenesis is allowed to proceed for the residence time in that step (all steps are of equal duration). The amount of methane and cells produced is calculated based on the relationship of biological methanogenesis to temperature and hydrogen concentration derived from laboratory bottle experiments (temperature) and continuous flow chemostat experiments (hydrogen). The residence time and starting cell concentration are varied to produce outcomes which match the methane, hydrogen, and methanogen concentrations measured in the field. These represent the possible range of residence times and cell concentrations for methanogens in the subsurface at these vent sites.

The main conclusion from the model currently is that shorter residence times in the subsurface for hydrothermal fluid favor methanogens with lower optimum temperatures. If residence times are long – either because fluid flow rate is slow or the distance travelled is long – the dilute fluid remains at higher temperatures long enough for hyperthermophilic methanogens to consume most or all of the available hydrogen. At fast flow rates or where the distance travelled is short, hydrogen remains unconsumed and is available for use by thermophilic methanogens, until the seawater intrusion raises the oxygen concentration to a point where methanogenesis is no longer sustainable (as methanogens are obligate anaerobes.) Given this, our three field sites represent three different scenarios for methanogenesis at Axial. At Anemone, hydrogen concentrations are so low that methanogenesis is either impossible or only possible in microenvironments, which is reflected in both the molecular and culture-based concentrations of methanogens – almost or entirely absent. At Marker 33, residence times in the subsurface are relatively long, allowing hyperthermophilic methanogens to



consume a significant portion of hydrogen and accounting for their relative prominence among the methanogen population. However, hydrogen concentrations are still lower than at Marker 113, where methanogens dominate both the archaeal and overall prokaryotic populations. The methanogens which dominate at Marker 113 are primarily thermophilic, as residence time is presumably short and hydrogen is still in high concentrations at thermophilic temperatures.

This model has a number of features that make it an imprecise reflection of the likely real situation in the subsurface at Axial. Firstly, it assumes that all hydrogenotrophy is conducted by methanogens, although other hydrogenotrophic organisms are known to exist at these vents. Secondly, it does not incorporate measurements of fluid flow, so we cannot predict whether residence times are made longer by fluid flow rates or the distance from ‘source’ hydrothermal fluid to the seafloor. Thirdly, it is not a physically accurate model of fluid flow through hard rock, as it assumes a single path of flow through a “tube”. Fourthly, it assumes that residence time at each step is equal, and that equal amounts of seawater are added to hydrothermal fluid flow at each step, which affects predictions of residence time. Finally, it does not account for either hydrogen production by heterotrophic organisms or methane consumption by methanotrophs, both of which could mask the real extent of methanogenesis in this system. Ultimately, however, all models contain assumptions that make them inaccurate to some degree compared to the complexities of the systems they represent. This model still represents the first attempt to apply well-understood strategies for understanding microbial activity in subsurface terrestrial aquifers to a subsurface ocean crust aquifer, and it can be brought closer and

closer to the complexities of the real system by the iterative addition of the components measured above, as the appropriate data become available to incorporate.

# CHAPTER 3

## THERMOPHILIC, CHEMOLITHOAUTOTROPHIC SULFUR- REDUCING *DESULFUROBACTERIUM* SP. KINETICALLY OUTCOMPETES METHANOGENS FROM DEEP-SEA HYDROTHERMAL VENTS FOR HYDROGEN

### 3.1 Abstract

Hydrothermal fluids (341°C and 19°C) were collected from the Boardwalk metal-sulfide edifice at the Endeavour Segment in the northeastern Pacific Ocean to study anaerobic microbial growth in hydrothermal mineral deposits. Calculations of thermodynamic energy in end-member Boardwalk vent fluids mixed with seawater indicate the energy available for anaerobic redox reactions is very low ( $< 2 \text{ J kg}^{-1}$  mixed vent fluid) due to  $\text{H}_2$  limitation. A thermophilic, hydrogenotrophic bacterium, *Desulfurobacterium* strain HR11, and a thermophilic methanogen, *Methanothermococcus* strain BW11, were isolated from the 19°C fluid. Strain HR11 grows at 40-77°C ( $T_{\text{opt}}$  72-75°C), pH 5-8.5 ( $\text{pH}_{\text{opt}}$  6-7), and 1-5% (wt vol<sup>-1</sup>) NaCl ( $\text{NaCl}_{\text{opt}}$  3-4%). Highest growth rates occur when  $\text{S}_2\text{O}_3^{2-}$  and  $\text{S}^\circ$  are reduced to  $\text{H}_2\text{S}$ . Modest growth occurs by  $\text{NO}_3^-$  reduction. Monod constants for its growth are  $K_s$  of 30  $\mu\text{M}$  for  $\text{H}_2$  and  $K_s$  of 20  $\mu\text{M}$  for  $\text{S}_2\text{O}_3^{2-}$  with a  $\mu_{\text{max}}$  of 2.0  $\text{h}^{-1}$ . The minimum  $\text{H}_2$  and  $\text{S}_2\text{O}_3^{2-}$  concentrations for growth are 3  $\mu\text{M}$  and 5  $\mu\text{M}$ , respectively. A comparison of  $\text{H}_2$  Monod growth kinetics for strain HR11 and high-temperature methanogens suggests strain HR11 outcompetes methanogens for  $\text{H}_2$  if  $\text{S}_2\text{O}_3^{2-}$  and  $\text{S}^\circ$ , which come from pyrite weathering and abiotic sulfide oxidation by dissolved  $\text{O}_2$ , are not limiting.

### 3.2 Introduction

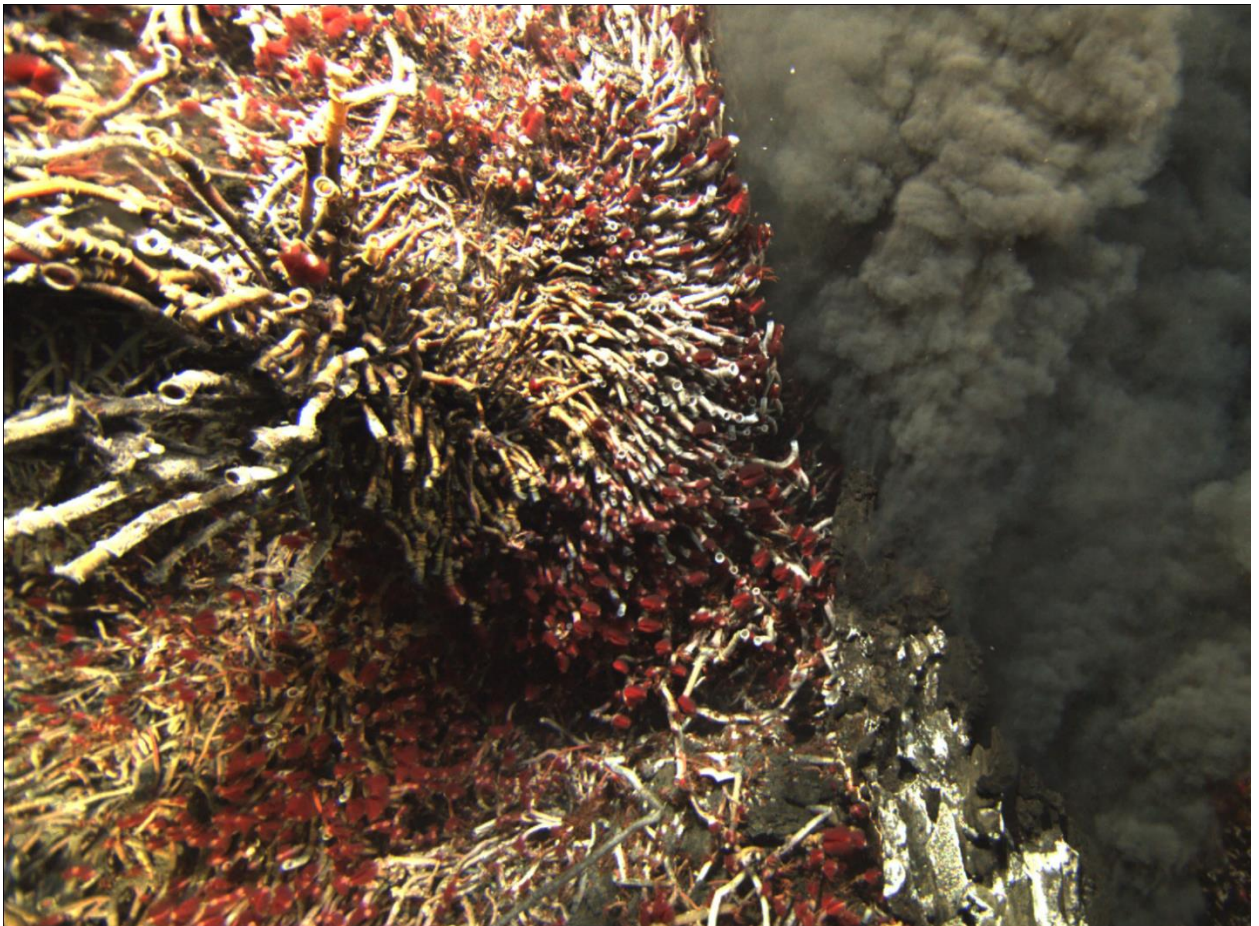
Deep-sea hydrothermal vents are seafloor expressions of biogeochemical processes that occur deeper within the rocky portions of the subseafloor (Deming and Baross, 1993; Orcutt *et al.*, 2011). Based on thermodynamic predictions of the energy available for redox reactions in mixtures of hydrothermal fluids and seawater, chemolithoautotrophy is generally dominated by aerobic H<sub>2</sub>S oxidation at mesophilic growth temperatures (e.g., below 50°C) and by anaerobic H<sub>2</sub> oxidation at higher temperatures at most hydrothermal vents (McCollom and Shock, 1997; Amend *et al.*, 2011). The amount of H<sub>2</sub> available for growth in hydrothermal fluids varies significantly based on host rock composition and frequency of volcanic activity (for summaries see Von Damm, 1995; Amend *et al.*, 2011; Holden *et al.*, 2012). The *Methanococcales* and the *Aquificales* are among the more common H<sub>2</sub>-oxidizing autotrophs found in hydrothermal vents (Huber and Holden, 2008). The *Methanococcales* are mesophilic-to-hyperthermophilic methanogens that are generally obligate hydrogenotrophs, although a few can also use formate (Whitman and Jeanthon, 2006). The *Aquificales* are strictly autotrophic and largely thermophilic H<sub>2</sub> oxidizers that use various sulfur compounds, NO<sub>3</sub><sup>-</sup>, and O<sub>2</sub> as electron acceptors (Huber and Eder, 2006). These organisms compete for H<sub>2</sub> between 40°C and 80°C.

In some anoxic environments such as freshwater sediments and sewage treatment plants, CH<sub>4</sub> formation is inhibited when SO<sub>4</sub><sup>2-</sup> concentrations are high (Lovley and Goodwin, 1988). Mesophilic sulfate-reducing bacteria (*Desulfovibrio*) have lower H<sub>2</sub> half-saturation constants for H<sub>2</sub> uptake and growth and higher maximum H<sub>2</sub> utilization and growth rates than mesophilic methanogens (*Methanobacterium*, *Methanobrevibacter*, *Methanospirillum* and *Methanosarcina*) (Kristjansson *et al.*, 1982; Lovley *et al.*, 1982;

Robinson and Tiedje, 1984; Karadagli and Rittmann, 2005). This enables sulfate reducers to inhibit CH<sub>4</sub> production by lowering the partial pressure of H<sub>2</sub> to concentrations below levels that methanogens can utilize. This is in keeping with the traditional hierarchy of anaerobic metabolisms, in which methanogenesis occurs only when all other electron acceptors are absent (Lovley and Goodwin, 1988). However, methanogens can co-exist with sulfate-reducing bacteria in the presence of SO<sub>4</sub><sup>2-</sup> where the outcome of competition is a function of the rate of H<sub>2</sub> supply, relative population sizes, and SO<sub>4</sub><sup>2-</sup> availability (Lovley *et al.*, 1982).

The purpose of this study is to assess the ability of thermophilic autotrophs to compete for H<sub>2</sub> in marine environments. Few measurements of H<sub>2</sub> growth kinetics have been made for autotrophic thermophiles. The minimum and Monod half-saturation (K<sub>s</sub>) H<sub>2</sub> values for the growth of deep-sea methanogens (*Methanocaldococcus*) at 70°C and 82°C were 17-23 µM and 67 µM, respectively (Ver Eecke *et al.*, 2012). In this study, an obligately hydrogenotrophic, thermophilic bacterium, *Desulfurobacterium* strain HR11, a member of the *Aquificales* that reduces S<sub>2</sub>O<sub>3</sub><sup>2-</sup>, S<sup>0</sup>, and NO<sub>3</sub><sup>-</sup>, and a thermophilic methanogen, *Methanothermococcus* strain BW11, were isolated from 19°C fluid flowing from the top of the Boardwalk hydrothermal edifice along the Endeavour Segment in the northeastern Pacific Ocean. This deposit was also venting 341°C hydrothermal fluid less than a meter away from a black-smoker chimney (Fig. 1). The physiological characteristics of strain HR11 and its minimum and K<sub>s</sub> values for growth on H<sub>2</sub> and S<sub>2</sub>O<sub>3</sub><sup>2-</sup> were measured and compared with those of high-temperature marine methanogens. Predictions are also made for the redox reaction energy available for

chemolithoautotrophic metabolism using various mixing ratios of the 341°C Boardwalk hydrothermal fluid and seawater.



**Figure 3.1. Boardwalk hydrothermal vent sampling site showing the black smoker (bottom) that was the source of the 341°C hydrothermal fluid and the tubeworm mound (left side) that was the source of the 19°C fluid. The image is a video frame grab from ROV Jason dive J2-576 at 18:46:15 on 07/22/2011.**

### 3.3 Materials and Methods

#### 3.3.1 Field sampling and redox energy estimates

In July 2011, 19°C and 341°C hydrothermal fluids were collected within a meter of each other on top of the Boardwalk hydrothermal edifice (Figure 3.1) at a depth of 2,134 m in the High Rise vent field along the Endeavour Segment of the Juan de Fuca Ridge (47.968°N 129.087°W). For the 341°C fluids, duplicate samples were drawn into Tedlar plastic bags with valves within rigid housings using the NOAA Hydrothermal Fluid and Particle Sampler (Butterfield *et al.*, 1997) and titanium gas-tight syringes (Edmond *et al.*, 1992). For the 19°C fluid, the sample was drawn into another Tedlar plastic bag. The sampler pumped vent fluid through a titanium nozzle and measured the temperature of the fluid at 1 Hz just inside the nozzle. Samples were collected using the remotely-operated vehicle (ROV) *Jason II* operated from the research vessel *Thomas G. Thompson*. Tedlar bag sample valves were closed on arrival on deck, and samples were stored under refrigeration until processed. Fluid samples were analyzed on board ship for pH, alkalinity, H<sub>2</sub>S, dissolved silica, and NH<sub>3</sub>. The gases were extracted from the gas-tight syringes using a shipboard gas extraction line and sealed in glass ampules for later analysis by gas chromatography. The extraction water (acidified with sulfamic acid) was analyzed for major elements on shore. Major and minor elements in the hydrothermal fluids were analyzed at the Pacific Marine Environmental Laboratory and at the University of Washington as described previously (Edmond *et al.*, 1992; Butterfield *et al.*, 1997).

Four redox reactions were considered for microbial energy availability estimates. Two represent aerobic respiration of inorganic electron donors (sulfide and methane oxidation) and two represent anaerobic respiration of H<sub>2</sub> and inorganic electron acceptors (sulfate reduction, methanogenesis) as previously described (Amend *et al.*, 2011). The compositions of the mixed hydrothermal solutions were calculated from those of the end-member vent fluid from Boardwalk and seawater using the REACT module in the computer code Geochemist's Workbench<sup>TM</sup> as previously described (Jin and Bethke, 2005; Amend *et al.*, 2011). All minerals were allowed to precipitate in the model during mixing except quartz, tridymite, cristobalite, chalcedony, and hematite (Jin and Bethke, 2005; Amend *et al.*, 2011). HS<sup>-</sup>, CH<sub>4(aq)</sub> and NH<sub>4</sub><sup>+</sup> were decoupled from redox reactions, but all other redox reactions were allowed. O<sub>2</sub> concentrations and predicted pH values given are from this model. Values of Gibbs energy ( $\Delta G_r$ ) for the catabolic reactions were computed using the activities of relevant species as previously described (Amend *et al.*, 2011). The amount of energy available (J) from catabolic reactions at 25°C, 37°C, 45°C, 55°C, 70°C, 85°C and 100°C in a kg of mixed fluid was calculated by multiplying the calculated Gibbs energy for the reaction at each temperature by the concentration of reactants in the mixed fluid (Amend *et al.*, 2011). These took into account the stoichiometry of the reaction and the reactant that was in limiting supply.

### 3.3.2 Isolation of new thermophile strains

Hydrothermal fluid from the 19°C sample was immediately used to inoculate Deutsche Sammlung von Mikroorganismen und Zellkulturen (abbreviated DSM) medium 399 and modified DSM medium 282 (see below) (Jones *et al.*, 1983; Burggraf, Jannasch, *et al.*, 1990) for the growth of autotrophic sulfur reducers and methanogens. The samples were



incubated shipboard at 55°C until they became turbid. Growth was confirmed using phase-contrast light microscopy, headspace analysis using gas chromatography, and sulfide production using the methylene blue method (Chen and Mortenson, 1977). Cells in the modified DSM 282 medium were predominantly rods that produced H<sub>2</sub>S and very little CH<sub>4</sub>, while those in the DSM 399 medium were predominantly cocci that produced CH<sub>4</sub>. On shore, purification of the strains from the 19°C hydrothermal fluid was performed by three rounds of 10-fold dilution-to-extinction incubations at 55°C using their original enrichment medium. The result was the purification of *Desulfurobacterium* strain HR11 from the modified DSM 282 medium and *Methanothermococcus* strain BW11 from the DSM 399 medium. *Desulfurobacterium thermolithotrophum* (DSM 11699) was purchased from DSM (Braunschweig, Germany) for comparison.

### 3.3.3 Cell characteristics

For *Desulfurobacterium* strain HR11 and *Methanothermococcus* BW11, growth and production of H<sub>2</sub>S and CH<sub>4</sub> on amended media were confirmed after three successive transfers on each medium. For kinetic experiments, at least 10 Balch tubes were inoculated concurrently with a logarithmic growth phase culture grown under the same experimental conditions. At various time points, at least two tubes were permanently removed from incubation. Cell concentrations and specific growth rates were determined as described above. For strain HR11, spectrometry was used to measure the amount of H<sub>2</sub>S by adding 0.1 N NaOH to each tube and then using the methylene blue method. For strain BW11, the volume of gas within each tube was measured with a pressure lock syringe. Gas chromatography was used to measure the amount of CH<sub>4</sub> in an aliquot of the headspace using a molecular sieve 5A column in a Shimadzu GC-17A gas

chromatograph with a flame-ionization detector. Cell yields relative to CH<sub>4</sub> produced ( $Y_{\text{CH}_4}$ ) were calculated from the best-fit linear slope of the number of cells per tube plotted against the amount of CH<sub>4</sub> per tube for each time point throughout the logarithmic phase of growth. Methane production rates ( $v$ ) were calculated as previously described (Ver Eecke *et al.*, 2013). Confidence intervals (95%) were calculated for growth rates, cell yields and metabolite production rates as described previously (Zar, 1996).

*Desulfurobacterium* strain HR11 was grown at pH 4 (no buffer), pH 5 and 6 (5 mM MES buffer), pH 7 and 8 (20 mM PIPES buffer), and pH 8.5 and 9 (30 mM NaHCO<sub>3</sub> buffer) to determine the effect of pH on growth. It was also grown on 0-5% (wt vol<sup>-1</sup>) NaCl to determine the effect of salt on growth. Elemental sulfur (0.2% wt vol<sup>-1</sup>), 10 mM NaSO<sub>3</sub>, 20 mM Na<sub>2</sub>SO<sub>4</sub>, 20 mM ferric citrate, 100 mmol L<sup>-1</sup> amorphous Fe(III) (oxy)hydroxide, and 20 mM KNO<sub>3</sub> were tested separately in place of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> as terminal electron acceptors. Yeast extract (0.2% wt vol<sup>-1</sup>), 10 mM maltose, 10 mM tryptone, 10 mM sodium acetate, and 10 mM sodium formate were tested separately as carbon and electron donors using 2 atm of N<sub>2</sub>/CO<sub>2</sub> (80:20 ratio) and H<sub>2</sub> only in the headspace. All tests were done in duplicate, and positive cultures were transferred three times on the same substrate to confirm growth.

#### 3.3.4 Phylogenetic analyses

DNA was extracted from strain HR11 and BW11 using a genomic DNA extraction kit (Qiagen) and their 16S rRNA genes were amplified using the polymerase chain reaction and sequenced. The primers used were 8f (5'-AGA GTT TGA TCC TGG CTC A-3') and 1492r (5'-TAC CTT GTT ACG ACT T-3'). Phylogenetic trees were constructed using 16S rRNA gene sequences from the Ribosomal Database Project (Cole *et al.*, 2007) in

MEGA 5 (Tamura *et al.*, 2011) using a neighbor-joining algorithm. Both sequences are deposited in GenBank under accession numbers KR023948-KR023949.

### 3.3.5 Electron microscopy

For negative staining of whole mounted cells, 10 ml of culture within a sealed Balch tube were fixed by adding 0.2 ml of 50% glutaraldehyde with gentle mixing and incubating at room temperature for 1 h. An aliquot (3 ml) of the fixed culture was then removed from the sealed Balch tube, processed, and applied to plasma-treated carbon films (ca. 0.5 nm thickness) on 400 mesh copper grids. The grids were stained with 3% NH<sub>4</sub>OH and 2% aqueous uranyl acetate and viewed with a JEOL-100S transmission electron microscope. For thin section microscopy, 10 ml of culture within a sealed Balch tube were fixed by adding 0.4 ml of 50% glutaraldehyde with gentle mixing and incubating at room temperature for 2 h. An aliquot (3 ml) of the fixed culture was then removed from the sealed Balch tube, post fixed, and enrobed by resuspension in a minimal volume of 2% type IX agarose to create a non-friable unit rich in cells. The agarose was then gelled and cut into 1 mm blocks with a razor blade. These blocks were then rinsed in dH<sub>2</sub>O, dehydrated, infiltrated with Ellis-Spurrs low-viscosity epoxy resin formulation (Ellis, 2006). Polymerized blocks were sectioned on a diamond knife set at 60 nm thickness. Sections were stained with 2% aqueous uranyl acetate and alkaline lead citrate (5 mg ml<sup>-1</sup> in 0.1 N NaOH). Sections were viewed on a JEOL 100S transmission electron microscope.

### 3.3.6 Growth conditions

The growth medium for all laboratory experiments, except where amended as described, was DSM medium 282 that was modified by the addition of 0.1% (wt vol<sup>-1</sup>) Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, the

removal of  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , and the use of 6.4 mM dithiothreitol (DTT) as the reducing agent instead of  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  and cysteine-HCl (Jones *et al.*, 1983). DTT was initially used to permit measurement of sulfide production and in other experiments for consistency. The medium was pH balanced to  $6.00 \pm 0.05$ . Static cultures were grown in 10 ml of medium contained within Balch tubes sealed with butyl rubber stoppers with 2 atm of  $\text{H}_2/\text{CO}_2$  (80:20 ratio) headspace and incubated in a forced-air incubator. Strain HR11 was incubated at  $72^\circ\text{C}$  unless otherwise indicated.

For the  $\text{H}_2$  and  $\text{Na}_2\text{S}_2\text{O}_3$  limitation experiments, a 2-L bioreactor stirred at 300 rpm with controls for gas flow, incubation temperature ( $72^\circ\text{C} \pm 0.1^\circ\text{C}$ ), and pH ( $6.0 \pm 0.1$  by the automatic addition of 0.25 mM HCl) was prepared with 1.5 L of modified DSM 282 medium. The reactor was degassed through a submerged fritted bubbler with a mixture of  $\text{CO}_2$  (7.5 mL of gas  $\text{min}^{-1}$ ),  $\text{H}_2$ , and  $\text{N}_2$ . For the  $\text{H}_2$  limitation experiments, the  $\text{H}_2$  gas flow rate and  $\text{H}_2$  concentration were varied for different growth kinetics experiments. A  $\text{H}_2/\text{N}_2$  tank mixture (5:95 ratio) was used in place of pure  $\text{H}_2$  to attain  $\text{H}_2$  concentrations below 20  $\mu\text{M}$ .  $\text{N}_2$  was added to balance the total gas flow at 70  $\text{mL min}^{-1}$ . The aqueous  $\text{H}_2$  concentration in the reactor at all  $\text{H}_2$  flow rate settings was measured by drawing ~ 25 mL of fluid from the bottom of the reactor directly into anoxic 60-mL serum bottles and measuring the headspace in the bottle with a gas chromatograph. For the  $\text{Na}_2\text{S}_2\text{O}_3$  limitation experiments, the given concentration is for the initial concentration in the reactor. The reactor was inoculated with a logarithmic growth-phase culture of strain HR11. During growth, samples were drawn from the reactor and cell concentrations were determined using phase-contrast light microscopy and a Petroff-Hauser counting chamber. Specific growth rates ( $\mu$ ) were estimated using a best-fit curve through the

exponential portion of growth. Each growth kinetic experiment was run in duplicate. Minimum H<sub>2</sub> requirements are given as the H<sub>2</sub> concentration below which organisms would not reliably grow in batch bioreactor culture.

### **3.4 Results**

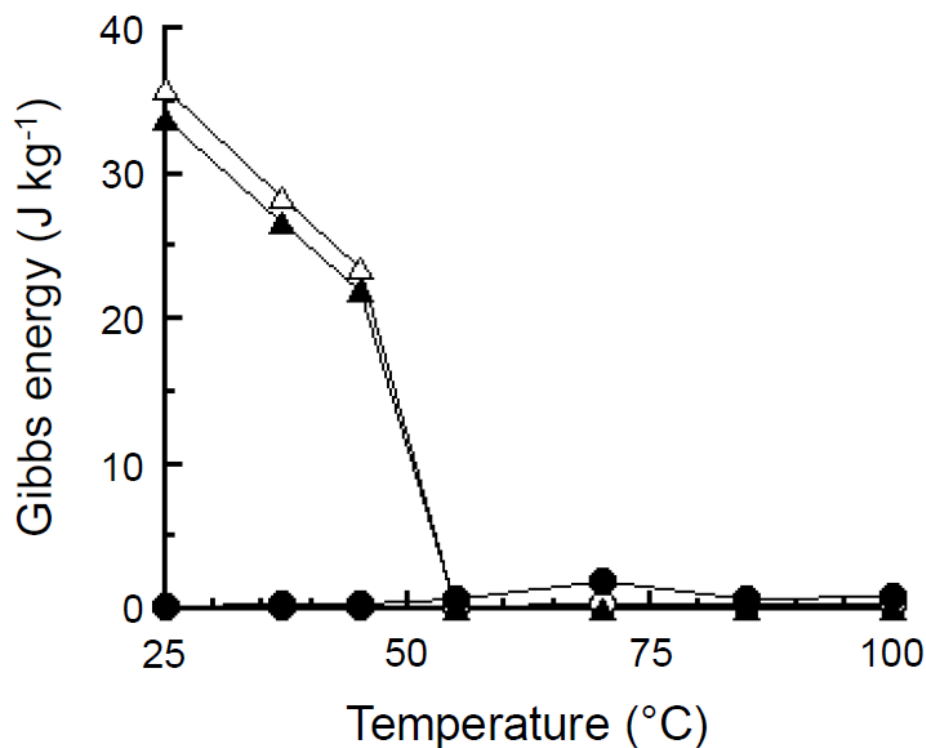
#### **3.4.1 Fluid chemistry and reaction energetics**

Most of the calculated end-member chemical concentrations for the hydrothermal fluids emanating from the Boardwalk sulfide chimney (Table 3.1) fall within the range of previously measured values for Endeavour Segment hydrothermal fluids (Lilley *et al.*, 1993; Butterfield *et al.*, 1994; Lilley *et al.*, 2003). The pH of the end-member fluid was mildly acidic (pH 4.1) when measured at 25°C. Hydrogen concentrations were low relative to historical values for Endeavour (Lilley *et al.*, 1993; Butterfield *et al.*, 1994; Lilley *et al.*, 2003; Ver Eecke *et al.*, 2012). Methane and NH<sub>4</sub><sup>+</sup> concentrations were typical for Endeavour, but highly elevated relative to global mid-ocean ridge hydrothermal systems (Von Damm, 1995; Holden *et al.*, 2012). The concentrations of Cl<sup>-</sup> and the major cations (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>) were above that of seawater, reflecting minor brine enrichment (Butterfield *et al.*, 1994; Von Damm, 1995; Butterfield *et al.*, 1997).

	Hydrothermal fluid	Seawater <sup>a</sup>
Temperature, max.	341°C	2°C
pH at 25°C	4.1	7.8
H <sub>2</sub> (μmol kg <sup>-1</sup> )	79	0
CH <sub>4</sub> (μmol kg <sup>-1</sup> )	2,680	0
O <sub>2</sub> (μmol kg <sup>-1</sup> )	0	123
Na <sup>+</sup> (mmol kg <sup>-1</sup> )	506.9	441
K <sup>+</sup> (mmol kg <sup>-1</sup> )	36.2	9.8
NH <sub>4</sub> <sup>+</sup> (μmol kg <sup>-1</sup> )	833	-
Mg <sup>2+</sup> (mmol kg <sup>-1</sup> )	0.01	54.5
Ca <sup>2+</sup> (mmol kg <sup>-1</sup> )	48.2	10.7
Fe <sup>2+</sup> (μmol kg <sup>-1</sup> )	1,300.4	0
Cl <sup>-</sup> (mmol kg <sup>-1</sup> )	621.9	550
SO <sub>4</sub> <sup>2-</sup> (mmol kg <sup>-1</sup> )	1.7	27.9
HCO <sub>3</sub> <sup>-</sup> (mmol kg <sup>-1</sup> )	29.4	2.2
HS <sup>-</sup> (mmol kg <sup>-1</sup> )	3.4	0
SiO <sub>2</sub> (mmol kg <sup>-1</sup> )	18.1	0.13

**Table 3.1: Chemical composition of end-member hydrothermal vent fluid from the Boardwalk edifice extrapolated to zero-Mg<sup>2+</sup> from this study and seawater for modeling purposes <sup>a</sup>Seawater composition from Amend *et al.* (2011), except the O<sub>2</sub> concentration which is from Von Damm *et al.* (1985).**

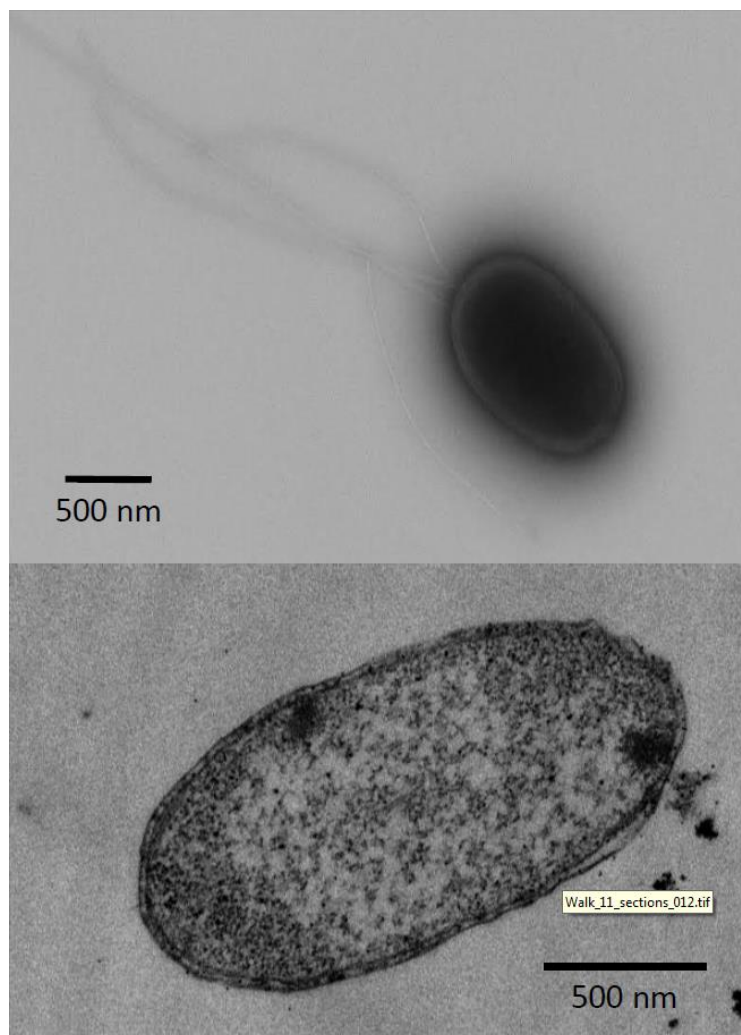
At 25-45°C, aerobic sulfide oxidation and methane oxidation provide the largest amount of redox energy (22.0-35.8 J kg<sup>-1</sup> mixed vent fluid) for microbial catabolism (Figure 3.2). They are both limited by the availability of O<sub>2</sub> in seawater. Between 45°C and 55°C, O<sub>2</sub> is predicted by the mixing model to be depleted in the mixed hydrothermal fluid. Among hydrogenotrophic reactions, the energies for sulfate reduction (0.12-1.85 J kg<sup>-1</sup> mixed vent fluid) and methanogenesis (0.12-0.38 J kg<sup>-1</sup> mixed vent fluid) increase with temperature due to the increased availability of H<sub>2</sub>, but are substantially lower than the reaction energies available for mesophilic, aerobic metabolism (Figure 3.2). The pH of the mixed vent fluid is predicted by the mixing model to be pH 4.1 at 100°C, pH 4.9 at 55°C, and pH 5.7 at 25°C.



**Figure 3.2: Predicted catabolic energies (per kg of mixed fluid) available for hydrogenotrophic sulfate reduction (●), hydrogenotrophic methanogenesis (○), aerobic sulfide oxidation (▲), and aerobic methane oxidation (Δ) at varying temperatures in mixed abiotic hydrothermal-seawater solutions flowing from the Boardwalk edifice.**

### 3.4.2 Characteristics of strains HR11 and BW11

Phylogenetically, strain HR11 is closely related (>99% identity) to *Desulfurobacterium thermolithotrophum* (L'Haridon *et al.*, 1998) and strain BW11 is closely related (>97% identity) to *Methanothermococcus okinawensis* (Takai *et al.*, 2002) (Figure 3.4). Electron microscopy of strain HR11 reveals short oblong rods, 0.5  $\mu\text{m}$  by 1-2  $\mu\text{m}$ , with a typical Gram-negative bacterial cell envelope and lophotrichous flagellation with three flagella (Figure 3.3).

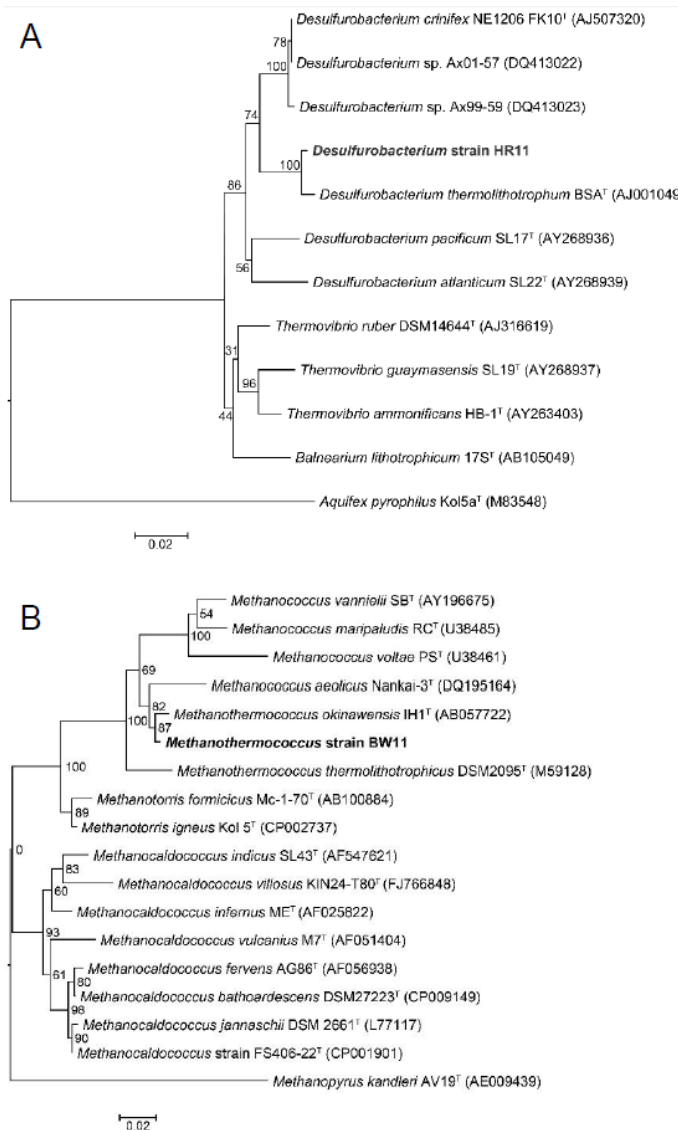


**Figure 3.3: Negative staining (A) and thin-section transmission electron micrographs of strain HR11. Bars, 500 nm.**

Growth is observed between 40°C and 77°C with an optimum of 72-75°C (Figure 3.5A), between pH 5.0 and 8.5 with an optimum of pH 6.0-7.0 (Figure 3.5B), and between 1% and 5% NaCl with an optimum of 3-4% (Figure 3.5C). Metabolite measurements show that the organism produces up to 6 mM H<sub>2</sub>S. Strain HR11 is an obligate hydrogenotrophic autotroph that does not utilize yeast extract, maltose, tryptone, acetate or formate as an alternative source of carbon or electrons. It grows at the same rate on elemental sulfur ( $1.56 \pm 0.17 \text{ h}^{-1}$ ) as it does on Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> ( $1.59 \pm 0.26 \text{ h}^{-1}$ ), and shows modest growth ( $0.24 \pm 0.21 \text{ h}^{-1}$ ) when KNO<sub>3</sub> is the terminal electron acceptor. Strain

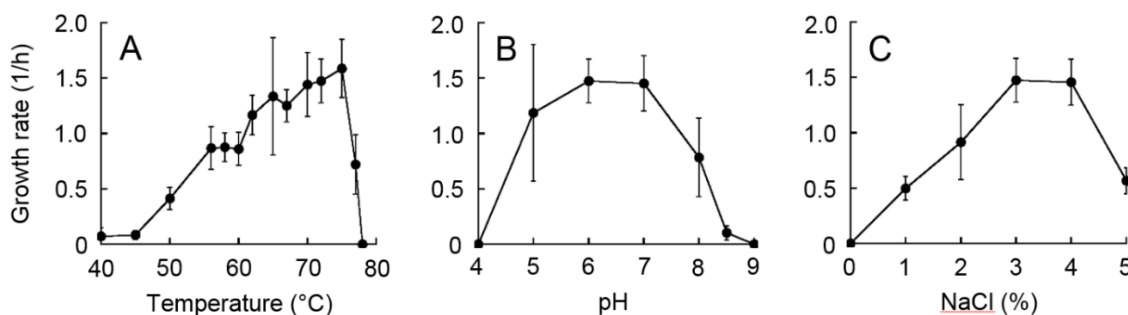


HR11 does not grow when  $\text{Na}_2\text{SO}_3$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{Fe(III)-citrate}$ , or  $\text{Fe(III) (oxy)hydroxide}$  are used as the terminal electron acceptor. The specific growth rate of *D. thermolithotrophum* on modified DSM 282 medium is significantly lower ( $0.23 \pm 0.07 \text{ h}^{-1}$ ) than that of strain HR11 at the same temperature.



**Figure 3.4: Neighbor-joining trees showing the positions of (A) strain HR11 within the genus *Desulfurobacterium* (870 nt) and (B) strain BW11 within the genus *Methanothermococcus* (880 nt) based on sequences of the 16S rRNA gene. GenBank/EMBL/DBJ accession numbers are included in parentheses. The topology of the tree was estimated by bootstraps based on 500 replications. Numbers at the branch point are the percentage support by bootstraps. Bar, 2% sequence divergence.**

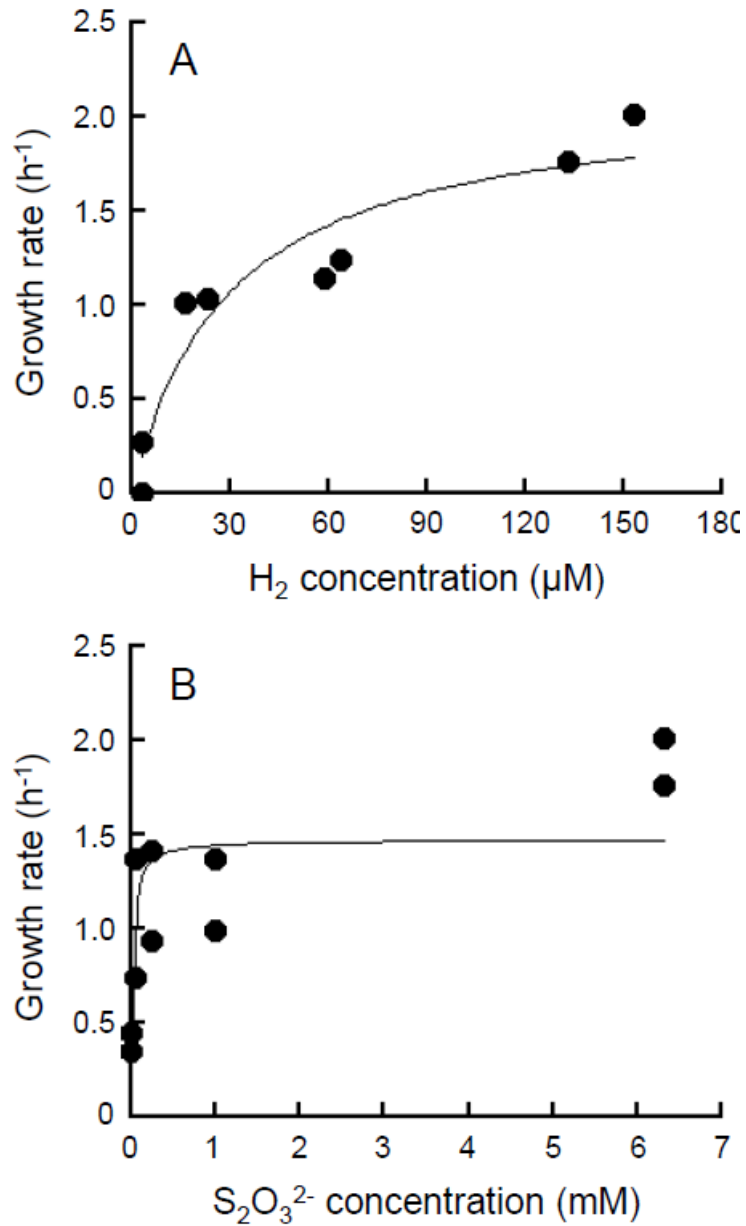
*Methanothermococcus* strain BW11 grows between 35°C and 75°C with an optimum of 65°C ( $\mu = 0.89 \pm 0.20 \text{ h}^{-1}$ ). Its growth rates are the same on DSM 399 medium and modified DSM 282 medium. Cell growth yields with respect to CH<sub>4</sub> production remain constant across its growth temperature range ( $7.6\text{--}8.1 \times 10^{12} \text{ cells mol}^{-1} \text{ CH}_4$ ). Its CH<sub>4</sub> production rate increases with increasing growth temperature from  $23.2 \pm 5.5 \text{ fmol CH}_4 \text{ cell}^{-1} \text{ h}^{-1}$  at 35°C to  $166.1 \pm 42.2 \text{ fmol CH}_4 \text{ cell}^{-1} \text{ h}^{-1}$  at 65°C.



**Figure 3.5: Growth rates for strain HR11 grown over its ranges of temperature (A), pH (B), and NaCl concentration (C). Error bars represent 95% confidence intervals.**

### 3.4.3 Monod kinetics for *Desulfurobacterium* strain HR11

*Desulfurobacterium* strain HR11 was grown in a gas flow-controlled bioreactor at 72°C to determine the effect of H<sub>2</sub> and S<sub>2</sub>O<sub>3</sub><sup>2-</sup> concentration on growth. It has longer doubling times and lower maximum cell concentrations with decreasing H<sub>2</sub> and S<sub>2</sub>O<sub>3</sub><sup>2-</sup> concentrations. The minimum H<sub>2</sub> concentration for growth is 3 μM and the K<sub>s</sub> for growth on H<sub>2</sub> is 30 μM (Figure 3.6A). When grown on excess H<sub>2</sub> (>100 μM), strain HR11 grows on as little as 5 μM S<sub>2</sub>O<sub>3</sub><sup>2-</sup> and its K<sub>s</sub> for growth is 20 μM (Figure 3.6B). The maximum growth rate ( $\mu_{\max}$ ) in the reactor is 2.0 h<sup>-1</sup>.



**Figure 3.6: Growth rates for strain HR11 grown over its ranges of H<sub>2</sub> concentration (A) and initial Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> concentration (B). The line is a Michaelis-Menten/Monod fit to the data ( $v = (v_{\max} * [H_2]) / (k_{H_2} + [H_2])$ ), where  $v$  = growth rate and  $k = [H_2]$  when  $v = 1/2 v_{\max}$ .**

### 3.5 Discussion

Hydrogen concentrations in most of the pure (zero-Mg<sup>2+</sup>) hydrothermal fluids from the Endeavour Segment since 2008 have been below 100 μmol kg<sup>-1</sup> (Ver Eecke *et al.*, 2012), which peaked in some vents at >1 mmol kg<sup>-1</sup> in 1999 following seismic activity (Lilley *et*

*al.*, 2003). For the Boardwalk edifice in 2011, diluting the 341°C end-member hydrothermal fluid with seawater to 40-75°C results in H<sub>2</sub> concentrations between 9 and 17 µM in the mixed fluid. These concentrations are at or near the minimum necessary for the growth of *Desulfurobacterium* strain HR11 and various hyperthermophilic methanogens (Ver Eecke *et al.*, 2012). The redox energy output of hydrogenotrophic sulfate reduction and methanogenesis at these temperatures is ~100-fold lower than the energy available for aerobic sulfide and methane oxidation at 25°C. The energy outputs of methanogenesis and hydrogenotrophic sulfate reduction are comparable to each other, with only a slight advantage to sulfur reducers. The standard Gibbs energy ( $\Delta G_r^\circ$ ) for hydrogenotrophic thiosulfate reduction is comparable to that for sulfate reduction while that of elemental sulfur reduction is reduced by ~ 40% (Amend and Shock, 2001). However, the redox energy available for thiosulfate and sulfur reduction will be lower than that of sulfate reduction if thiosulfate and sulfur availability is lower than sulfate availability. So the redox reaction energy for ‘sulfur’ reducers and methanogens is very low relative to aerobic reactions and the same reactions in high H<sub>2</sub> environments, as previously reported (Amend *et al.*, 2011). Yet thermophilic, obligately-hydrogenotrophic thiosulfate/sulfur reducers and methanogens are continuously flushed from the Boardwalk edifice. These organisms must reproduce in order to maintain their populations. This raises questions about the constraints on the growth of hydrogenotrophic thermophiles in this and similar low-H<sub>2</sub> hydrothermal environments, and about their ability to compete for H<sub>2</sub>.

The optimal growth temperature of *Desulfurobacterium* strain HR11 (72°C) is higher than that of *Methanothermococcus* strain BW11 (65°C) and lower than those of *Methanocaldococcus* spp. (80-85°C) (Whitman and Jeanthon, 2006). Ver Eecke *et al.* (2012) previously measured the minimum and  $K_s$  values of  $H_2$  for the growth of three *Methanocaldococcus* species grown at 70°C and 82°C. All three organisms had minimum  $H_2$  requirements of 17-23  $\mu M$ , a  $K_s$  for  $H_2$  of 67  $\mu M$ , and a  $\mu_{max}$  of 0.8-1.2  $h^{-1}$ . (When comparing to vent  $H_2$  values, we assume that  $\mu M$  in lab conditions  $\sim \mu mol\ kg^{-1}$ , given the density of water at atmospheric pressure.) In this study, *Desulfurobacterium* strain HR11 has a lower minimum  $H_2$  requirement, a lower  $H_2$   $K_s$ , and a higher  $\mu_{max}$  than those reported for *Methanocaldococcus*. The  $\mu_{max}/K_s$  ratios for  $H_2$  indicate that *Desulfurobacterium* strain HR11 has a growth advantage over *Methanocaldococcus* species. Its growth rate in Balch tubes is significantly higher than that of *Methanothermococcus* strain BW11. In our 55°C cell enrichments,  $H_2S$  production exceeded  $CH_4$  production in a medium containing  $Na_2S_2O_3$ , whereas a thiosulfate-free medium had only  $CH_4$  production. Therefore, the data suggest *Desulfurobacterium* from Endeavour will outcompete methanogens for  $H_2$  as long as  $S_2O_3^{2-}$  or  $S^0$  is not limiting. However, this may not be universal among *Desulfurobacterium* species; *D. thermolithotrophum* grows more slowly in this and other studies (L'Haridon *et al.*, 1998) than strain BW11.

While there are no direct measurements of thiosulfate in hydrothermal vent fluids, thiosulfate is likely found in vent systems as an intermediate of metal-sulfide mineral weathering and  $HS^-$  oxidation. Submarine hydrothermal chimneys form when metal

sulfides precipitate from hot vent fluid upon mixing with cold seawater and through conductive cooling (Goldfarb *et al.*, 1983; Haymon, 1983; Kelley *et al.*, 2002). Chimney walls are commonly porous and permit the exchange of hydrothermal fluid and seawater through much of their interiors, creating temperature and chemical gradients between end member, hot hydrothermal fluids emitted from the interior and cold seawater (Tivey and Singh, 1997; Kristall *et al.*, 2006; Zhu *et al.*, 2007). Pyrite is abiotically oxidized by  $\text{Fe}^{3+}$ , which adsorbs to the pyrite and forms  $\text{Fe}^{2+}$  and  $\text{S}_2\text{O}_3^{2-}$ , although the  $\text{S}_2\text{O}_3^{2-}$  is rapidly oxidized to  $\text{SO}_4^{2-}$  if additional  $\text{Fe}^{3+}$  is present (Luther, 1987; Moses *et al.*, 1987). Pyrite is also oxidized by  $\text{O}_2$ . The reaction rate is ten-fold slower than  $\text{Fe}^{3+}$  as an oxidant, but  $\text{S}_2\text{O}_3^{2-}$  is present in higher concentrations due to its slow oxidation rate with  $\text{O}_2$  (Luther, 1987; Moses *et al.*, 1987). Thiosulfate is also a key intermediate of the oxidation of  $\text{HS}^-$  to  $\text{SO}_4^{2-}$ , especially where  $\text{O}_2$  concentrations are below saturation (Jørgensen, 1990). Therefore, *Desulfurobacterium* has a source of electron acceptors through the oxidation of metal sulfide minerals and dissolved  $\text{HS}^-$ , such as within a hydrothermal chimney at the oxic-anoxic interface.

For terrestrial mesophilic sulfate-reducing microbes, the Monod  $\text{H}_2$   $K_s$  is 2-4  $\mu\text{M}$  for *Desulfovibrio* strain G11 and 6-7  $\mu\text{M}$  for *Methanospirillum hungatei* JF-1 (Robinson and Tiedje, 1984). Similarly, the  $\text{H}_2$  uptake  $K_s$  is 1-2  $\mu\text{M}$  for five *Desulfovibrio* spp.; 3-7  $\mu\text{M}$  for *Methanobrevibacter*, *Methanobacterium* and *Methanospirillum* species; and 13  $\mu\text{M}$  for *Methanosarcina barkeri* strain MS (Kristjansson *et al.*, 1982; Robinson and Tiedje, 1984). These differences in substrate affinities, probably due to the energetic favorability of sulfate-reduction for the same amount of hydrogen versus methanogenesis, confer a

competitive advantage for sulfate-reducing bacteria over methanogens when  $\text{SO}_4^{2-}$  is not limiting. However, both groups of organisms coexist in anoxic environments when both  $\text{H}_2$  and  $\text{SO}_4^{2-}$  are plentiful (Lovley *et al.*, 1982).

A global survey of low-temperature hydrothermal fluids with co-localized phylogenetic and chemical analyses shows that *Desulfurobacterium* and the *Methanococcales* are both present in vent environments with  $\text{H}_2$  concentrations predicted to be above  $17\ \mu\text{M}$  at  $72^\circ\text{C}$ , and both are generally absent below this threshold (Table 3.2). This suggests that  $\text{S}_2\text{O}_3^{2-}$  or  $\text{S}^\circ$  are not at limiting concentrations in these systems, and that generally there is sufficient  $\text{H}_2$  flux in many vent systems to support both groups of organisms.

Our understanding of the diversity and distribution of microorganisms in hydrothermal vents, when different species cooperate or compete with each other, and the physiological mechanisms they use to accomplish these is still nascent. The diversity of thermophilic anaerobes in hydrothermal vents is relatively low, making pure cultures of these organisms useful for modeling these questions. Thermophilic methanogens such as *Methanothermococcus* and *Methanocaldococcus* spp. and autotrophic sulfur reducers such as *Desulfurobacterium* spp. are common in vent systems; grow over the same temperatures, pHs and salinities; and compete for  $\text{H}_2$ , making them ideal candidates for competition studies. Although *Desulfurobacterium* has a kinetic growth advantage over the *Methanococcales* as long as  $\text{S}_2\text{O}_3^{2-}$  or  $\text{S}^\circ$  is present, the two functional groups appear to coexist where the flux of  $\text{H}_2$  results in  $\text{H}_2$  concentrations of  $>17\ \mu\text{M}$ . An important

future research question is how these organisms respond physiologically to H<sub>2</sub> limitation and to each other during this limitation.



<b>Table 3.2: Characteristics of various global deep-sea hydrothermal vent sites and the presence or absence of <i>Desulfurobacterium</i> and <i>Methanococcales</i> species</b>													
	Boardwalk edifice, Endeavour Segment,	Hulk edifice, Endeavour Segment,	Axial Volcano, Juan de Fuca Ridge	Lucky Strike Field, Mid-Atlantic Ridge	Kilo Moana, Lau Basin	Mariner Field, Lau Basin	TOTO Caldera, Mariana Arc	Brothers Volcano, Kermadec Arc	Iheya North Field, Mid-Okinawa Trough	Kairei Field, Central Indian Ridge	Logatchev Field, Mid-Atlantic Ridge	Rainbow Field, Mid-Atlantic Ridge	
Geologic setting <sup>a</sup>	MORB	MORB	MORB	MORB	VA	VA	VA	VA	VA	UM	UM	UM	
Max. temp. (°C)	341	305	275	163-324	290-304	338-359	170	290	311	365	300-350	191-370	
H <sub>2</sub> S (mM) <sup>b</sup>	3.4	NA	37	2.4-3.4	3.5-3.9	6.1-19	14.6	7.9	4.0	4.0	0.3	1.8-3.3	
H <sub>2</sub> (μM) <sup>b</sup>	79	165	600	25-71	220-498	33-179	~10	17	~200	2,500	5,900	12,300-16,900	
H <sub>2</sub> (μM) at 72°C <sup>c</sup>	17	39	158	11-16	55-119	7-36	4	4	47	496	>1,400	>4,600	
<i>Desulfurobacterium</i>	+	+	+	–	+	–	–	–	+	+	+	+	
<i>Methanococcales</i>	+	+	+	–	+	+	–	–	+	+	+	+	
References <sup>d</sup>	1	2,3	4-6	7	8	8	9	10	11	12	13	7	

<sup>a</sup>Symbols and abbreviations: MORB, mid-ocean ridge basalt; VA, volcanic arc; UM, ultramafic; +, *Desulfurobacterium*- and *Methanococcales*- related 16S rRNA sequences found in the samples; n.d., none detected; NA, not available.

<sup>b</sup>End-member H<sub>2</sub> and CH<sub>4</sub> concentrations in hydrothermal fluids are based on an extrapolation of measured values to zero magnesium concentration.

<sup>c</sup>H<sub>2</sub> concentrations at 72°C are estimated assuming conserved mixing between 2°C seawater containing no H<sub>2</sub> and end-member hydrothermal fluid H<sub>2</sub>.

<sup>d</sup>References: 1, this study; 2, Anderson *et al.* (2013); 3, Ver Eecke *et al.* (2012); 4, Huber *et al.* (2002); 5, Huber *et al.* (2003); 6, Butterfield *et al.* (2004); 7, Flores *et al.* (2011); 8, Flores *et al.* (2012); 9, Nakagawa *et al.* (2006); 10, Takai *et al.* (2009); 11, Nakagawa *et al.* (2005); 12, Takai *et al.* (2004); and 13, Perner *et al.* (2007).

## CHAPTER 4

### TAXONOMY AND GENOME OF METHANOCALDOCOCCLUS BATHOARDESCENS SP. NOV.

#### 4.1 Abstract

A hyperthermophilic methanogen, strain JH146<sup>T</sup>, was isolated from 26°C hydrothermal vent fluid emanating from a crack in basaltic rock at Marker 113 vent, Axial Volcano in the northeastern Pacific Ocean. It is an obligate anaerobe that uses only H<sub>2</sub> and CO<sub>2</sub> for growth. Phylogenetic analysis based on 16S rRNA gene sequences showed that the strain is more than 97% similar to other species of the genus *Methanocaldococcus*. We sequenced the complete genome of this strain (1,607,556 bp) and analyzed it with a focus on its methane production via the acetyl-CoA pathway and its potential for biotechnological applications. Next, overall genome relatedness index analyses were performed to establish that strain JH146<sup>T</sup> is a novel species. For each analysis, strain JH146<sup>T</sup> was most similar to *Methanocaldococcus* sp. FS406-22, which can fix N<sub>2</sub> and also comes from Marker 113 vent. However, strain JH146<sup>T</sup> differs from strain FS406-22 in that it cannot fix N<sub>2</sub>. The average nucleotide identity score for strain JH146<sup>T</sup> was 87%, the genome-to-genome direct comparison score was 33-55%, and the species identification score was 93%. For each analysis, strain JH146<sup>T</sup> was below the species delineation cut-off. Full-genome gene synteny analysis showed that strain JH146<sup>T</sup> and strain FS406-22 have 97% genome synteny, but strain JH146<sup>T</sup> is missing the operons necessary for N<sub>2</sub> fixation and assimilatory nitrate reduction that are present in strain

FS406-22. Based on its whole genome sequence, strain JH146<sup>T</sup> is suggested to represent a novel species of the genus *Methanocaldococcus* for which the name *Methanocaldococcus bathoardescens* is proposed. The type strain is JH146<sup>T</sup> (=DSM 27223<sup>T</sup>=KACC 18232<sup>T</sup>).

## 4.2 Introduction

The genus *Methanocaldococcus* was proposed when the order *Methanococcales* was reclassified to reduce the diversity of each genus within the order (Boone *et al.*, 1993). It consists of strictly hyperthermophilic, hydrogenotrophic methanogens that are obligately anaerobic and coccoidal (Boone *et al.*, 1993; Whitman and Jeanthon, 2006). In this study, the complete genome sequence of a strain of the genus *Methanocaldococcus*, strain JH146<sup>T</sup>, was used to establish the novelty of the strain. The strain was isolated from 26°C hydrothermal fluid that was flowing from a crack in basaltic rock at Marker 113 vent at Axial Volcano on the Juan de Fuca Ridge in the northeastern Pacific Ocean (Ver Eecke *et al.*, 2012; 2013). It is an obligately anaerobic archaeon which uses only H<sub>2</sub> as its electron donor, and CO<sub>2</sub> as its sole electron acceptor and carbon source, producing CH<sub>4</sub> and H<sub>2</sub>O (Ver Eecke *et al.*, 2013). Strain JH146<sup>T</sup> grows within the temperature range of 58-90°C with an optimum temperature of 82°C, within the pH range of 4.5-9.0 with an optimum pH of 7.0, and within the chlorinity range of 127-974 mM chloride with an optimum chlorinity of 358 mM (Ver Eecke *et al.*, 2013). Its phenotypic characteristics are very similar to all other *Methanocaldococcus* species (Table 4.1). It differs from another closely related hyperthermophilic methanogen isolated from the same location, *Methanocaldococcus* sp. strain FS406-22, in that strain JH146 lacks the ability to fix N<sub>2</sub> (Mehta and Baross, 2006; Ver Eecke *et al.*, 2013).

### 4.3 Materials and Methods

#### 4.3.1 Genome sequencing

The genome sequence of *Methanocaldococcus* sp. strain JH146 was acquired using Roche 454 GS FLX Titanium and Illumina Hiseq 2000 (Macrogen, Korea) for hybrid sequencing and Newbler 2.3 assembler for genome assembly. The open reading frames (ORFs) were predicted by GeneMarkS (Besemer *et al.*, 2001), Glimmer 3.02 (Delcher *et al.*, 1999) and FgenesB (Softberry, Inc., Mount Kisco, NY). Their functions were verified using BLASTP (Altschul *et al.*, 1990) and InterProScan (Zdobnov and Apweiler, 2001). rRNAs and tRNAs were predicted using RNAmmer (Lagesen *et al.*, 2007) and tRNAscan-SE (Lowe and Eddy, 1997), respectively.

#### 4.3.2 Phylogenetic analysis

The phylogenetic relatedness of strain JH146<sup>T</sup> to other species of the genus *Methanocaldococcus* was determined using 16S rRNA gene sequences obtained from the Ribosomal Database Project (Cole *et al.*, 2007) and comparing them via megaBLAST (McGinnis and Madden, 2004). We aligned sequences representing all species of the order *Methanococcales* using the default settings for CLUSTAL W (Larkin *et al.*, 2007) in MEGA6 software (Tamura *et al.*, 2013). We reconstructed neighbor-joining phylogenetic trees in MEGA6 with the Jukes-Cantor model and bootstrap values obtained from 500 replicate trees (Figure 4.1). According to this alignment, the closest relatives to strain JH146<sup>T</sup> are *Methanocaldococcus* sp. FS406-22, *M. jannaschii* DSM 2661<sup>T</sup>, and *M. fervens* AG86<sup>T</sup>.

#### 4.3.3 Genome analysis

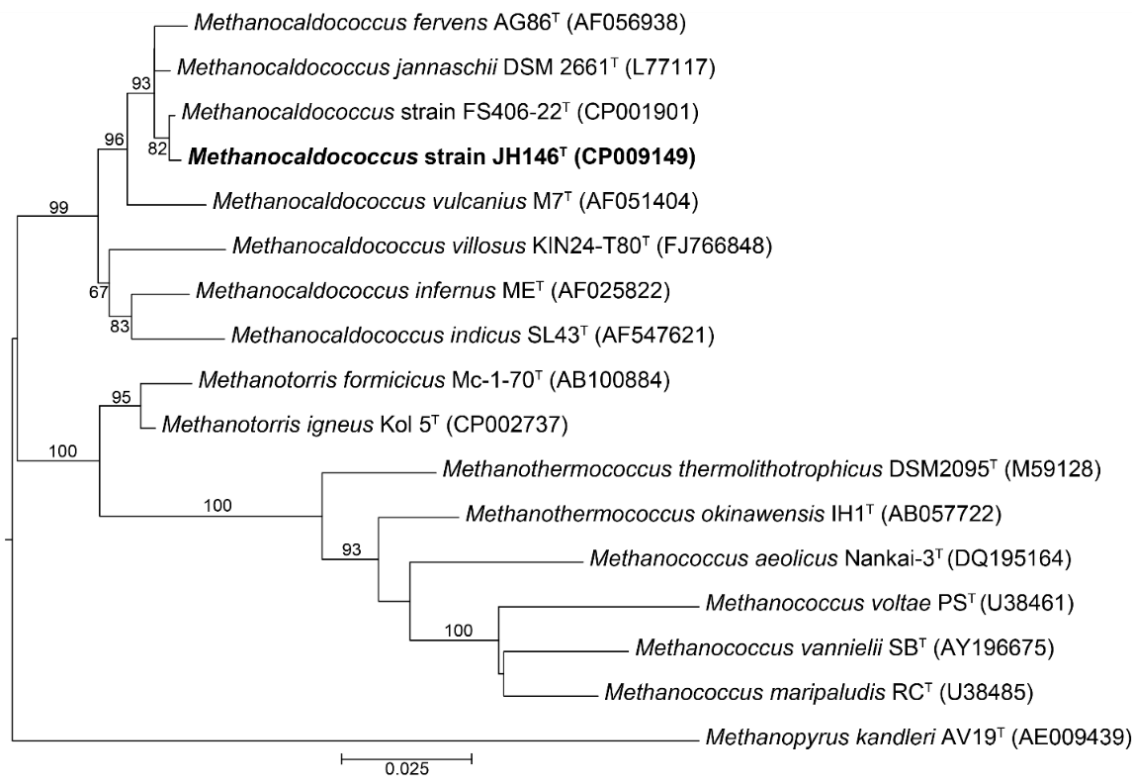
JH146<sup>T</sup> was compared with the complete genome sequences of their closest phylogenetic relatives and all other complete genome sequences of members of the genus *Methanocaldococcus* using overall genome relatedness index (OGRI) analyses (Chun and Rainey, 2014). All genome sequences were obtained from the GenBank sequence database (Table 4.2). We calculated the BLAST-based average nucleotide identity (ANI) score using the JSpecies program with the default parameters (Goris *et al.*, 2007; Richter and Rosselló-Móra, 2009). Genome-to-genome direct comparison (GGDC) analyses were performed using all three equations in the GGDC program, version 2.0 (Auch *et al.*, 2010). Forty marker genes were compared between strain JH146<sup>T</sup> and its closest relative, *Methanocaldococcus* sp. FS406-22, using the species identification (SpecI) program (Mende *et al.*, 2013). The program BLASTZ (Schwartz *et al.*, 2003) and the synteny mapping and analysis program (SyMAP version 4.0) (Soderlund *et al.*, 2011) were used to compute synteny blocks between strain JH146<sup>T</sup> and all other *Methanocaldococcus* genome sequences to determine which genes are present or absent in JH146<sup>T</sup> relative to its closest relatives.

### 4.4 Results and Discussion

#### 4.4.1 Phylogenetic tree

*Methanocaldococcus* strain JH146<sup>T</sup> showed 99.7% sequence identity with *Methanocaldococcus* sp. FS406-22, 99.3% identity with *Methanocaldococcus fervens* AG86<sup>T</sup>, 99.1% identity with *Methanocaldococcus jannaschii* DSM 2661<sup>T</sup>, 97.6% identity with *Methanocaldococcus vulcanius* M7<sup>T</sup>, and 97.0% identity with *Methanocaldococcus infernus* ME<sup>T</sup>. *Methanocaldococcus* sp. FS406-22 was also isolated from Marker 113 at

Axial Volcano (Mehta and Baross, 2006) but differs from strain JH146<sup>T</sup> in that only the former strain can fix N<sub>2</sub> (Ver Eecke *et al.*, 2013). According to the alignment shown in the phylogenetic tree below (Figure 4.1), the closest relatives to strain JH146<sup>T</sup> are *Methanocaldococcus* sp. FS406-22, *M. jannaschii* DSM 2661<sup>T</sup>, and *M. fervens* AG86<sup>T</sup>.



**Figure 4.1: Neighbor-joining tree showing the position of strain JH146<sup>T</sup> within the genus *Methanocaldococcus* based on sequences of the 16S rRNA gene (1293 nt). GenBank/EMBL/DBJ accession numbers are included in parentheses. The topology of the tree was estimated by bootstraps based on 500 replications. Numbers at the branch point are the percentage support by bootstraps. Bar, 2% sequence divergence.**

Characteristic	1	2	3	4	5	6	7	8
Temperature for growth (°C):								
Range	58-90	58-92	55-91	48-92	49-89	55-90	55-91	50-86
Optimum	82	90	85	85	80	80	85	85
pH for growth:								
Range	4.5-9.0	ND	5.2-7.0	5.5-7.6	5.25-7.0	5.5-7.0	5.25-7.0	5.5-6.7
Optimum	7.0	ND	6.0	6.5	6.5	6.5	6.5	6.5
NaCl for growth (%):								
Range	1.6-7.4	ND	1-5	0.5-5	0.6-5.6	0.5-5.5	1.25-5	1.5-5
Optimum	2.9	ND	2-3	3	2.5	2.5	2.5	3
No. of flagellar tufts	1	ND	2	ND	3	1	3	1
Resistance to rifampicin	+	ND	-	+	-	-	-	-

**Table 4.1: Differential characteristics of *Methanocaldococcus* species**

**Taxa: 1, strain JH146<sup>T</sup>; 2, strain FS406-22; 3, *Methanocaldococcus jannaschii* DSM2661<sup>T</sup>; 4, *Methanocaldococcus fervens* AG86<sup>T</sup>; 5, *Methanocaldococcus vulcanius* M7<sup>T</sup>; 6, *Methanocaldococcus villosus* KIN24-T80<sup>T</sup>; 7, *Methanocaldococcus infernus* ME<sup>T</sup>; 8, *Methanocaldococcus indicus* SL43<sup>T</sup>. Data were obtained from Jones *et al.* (1983), Zhao *et al.* (1988), Jeanthon *et al.*, (1998, 1999), L'Haridon *et al.* (2003), Mehta & Baross (2006), Bellack *et al.* (2010), and Ver Eecke *et al.* (2013). ND, not determined.**

#### 4.4.2 Genome similarity indices

Since the 16S rRNA gene sequences of species of the genus *Methanocaldococcus* generally show more than 97% identity across the genus (Figure 4.1), the complete genome sequences of strain JH146<sup>T</sup> were analyzed using Overall Genome Relatedness Indices (OGRI) to determine whether it was a novel species. For each OGRI analysis, strain JH146<sup>T</sup> was most closely related to *Methanocaldococcus* sp. FS406-22 (Table 4.2).

	ANI	GGDC	GGDC	GGDC
		DDH 1	DDH 2	DDH 3
<i>Methanocaldococcus</i> sp. strain FS406-22	<b>87</b>	<b>55</b>	<b>33</b>	<b>49</b>
<i>Methanocaldococcus jannaschii</i> DSM2661 <sup>T</sup>	87	54	33	48
<i>Methanocaldococcus fervens</i> AG86 <sup>T</sup>	85	45	30	40
<i>Methanocaldococcus infernus</i> ME <sup>T</sup>	79	14	23	14
<i>Methanocaldococcus vulcanius</i> M7 <sup>T</sup>	73	18	25	18

**Table 4.2: ANI and GGDC analyses of genomic DNA from strain JH146<sup>T</sup> and related species of the genus *Methanocaldococcus*. Data in bold type represent the closest relatives. DDH, DNA-DNA homology. GenBank/EMBL/DDBJ genome accession numbers: strain JH146<sup>T</sup>, CP009149.1; strain FS406-22, CP001901.1; *M. jannaschii* DSM2661<sup>T</sup>, L77117.1 (Bult *et al.*, 1996); *M. fervens* AG86<sup>T</sup>, CP001696.1; *M. infernus* ME<sup>T</sup>, CP002009.1; *M. vulcanius* M7<sup>T</sup>, CP001787.1.**

The ANI score for the strain comparison between JH146<sup>T</sup> and *Methanocaldococcus* sp. FS406-22 was 87%, which is below the 96% cut-off value for species determination by this approach. The GGDC calculations with BLAST+ for strain JH146<sup>T</sup> and *Methanocaldococcus* sp. FS406-22 gave DNA-DNA homology (DDH) values of 55, 33 and 49% for the three equations in the program, which are below the 60% cut-off for delineating species by this approach. The SpecI analysis for strain JH146<sup>T</sup> and *Methanocaldococcus* sp. FS406-22 gave an average identity of 92.5%, which is below the 96.5% cut-off for delineating species by this approach, with all 40 gene homologies below the species cut-off. All three OGRI analyses indicated that strain JH146<sup>T</sup> represents a novel species. Therefore, based on its whole genome sequence, strain JH146<sup>T</sup> is suggested to represent a novel species of the genus *Methanocaldococcus*, for which the name *Methanocaldococcus bathoardescens* sp. nov. is proposed.



#### 4.4.3 Notable genome features

The genome of *Methanocaldococcus* sp. strain JH146 consists of a circular chromosome of 1,607,556 bp with no extra chromosome (Table 4.3). It contains 1,668 ORFs and 16 pseudogenes with a GC content of 31.3%. The genome encodes 35 tRNAs and 6 rRNAs organized into two operons. It contains genes encoding various enzymes involved in the acetyl-CoA pathway for CO<sub>2</sub> reduction including formylmethanofuran dehydrogenase (JH146\_0111, JH146\_0255, JH146\_0654-0656, JH146\_0677-0681, and JH146\_1441-1445), formylmethanofuran-H<sub>4</sub>MPT formyltransferase (JH146\_0902), methenyl-H<sub>4</sub>MPT cyclohydrolase (JH146\_0543), and methylene-H<sub>4</sub>MPT reductase (JH146\_0140). It also contains the genes for H<sub>2</sub>-dependent (JH146\_1128, JH146\_1202) and F<sub>420</sub>-dependent (JH146\_0920) methylene-H<sub>4</sub>MPT dehydrogenase. The genes for methane production are methyl-H<sub>4</sub>MPT-CoM methyltransferase (JH146\_1065-1073), methyl-CoM reductase (JH146\_0494, JH146\_0514-0516, JH146\_0766, JH146\_1082-1086), and heterodisulfide reductase (JH146\_0660-0661, JH146\_1054-1055). The genome also contains genes for  $\alpha$ -amylase (JH146\_0025), glucoamylase/oligosaccharide amylase (JH146\_0026), starch synthase (JH146\_0029), and DNA polymerase (JH146\_0042, JH146\_0409, JH146\_0543, JH146\_1048, JH146\_1230 and JH146\_1381).

SyMAP analysis showed that strain JH146<sup>T</sup> shares 97% genome synteny with *Methanocaldococcus* sp. FS406-22 and 94% genome synteny with *M. jannaschii* DSM 2661<sup>T</sup>. JH146<sup>T</sup> and *M. jannaschii* DSM2661<sup>T</sup> lack the operons in FS406-22 which encode for nitrogen fixation (MFS40622\_0031 to MFS40622\_0035) and assimilatory nitrate reduction (MFS40622\_1410 to MFS40622\_1412). JH146<sup>T</sup> has sulfur-metabolism related proteins such as DsrE and DsrH (JH146\_1140 to JH146\_1143) that are missing in

FS406-22. JH146 has a number of chemotaxis-related proteins (JH146\_0928 to JH146\_0944) and CRISPR-associated proteins (JH146\_0426 to JH146\_0433) that are not present in both FS406-22 and *M. jannaschii* DSM2661<sup>T</sup>.

Attribute	Value
Genome size (bp)	1,607,556
DNA coding region (bp)	1,415,886
DNA G+C content	31.3%
Number of replicons	1
Total genes	1709
rRNA genes	6
tRNA genes	35
Protein coding genes	1650
Pseudogenes	18
Gene with predicted function	

**Table 4.3: Genome statistics**

#### 4.4.4 Description of *Methanocaldococcus bathoardescens*, sp. nov.

*Methanocaldococcus bathoardescens* (ba.tho.ar.des'cens. Gr. n. bathos depth; L. part. adj. ardescens becoming, hot, erupting (for a volcano); N.L. part. adj. bathoardescens, erupting in the depth).

Cells are lophotrichously flagellated irregular cocci. Diameter approximately 1-2 µm.

Obligate anaerobe. Optimal growth occurs at 82°C (range: 58°C to 90°C), pH 7.0 (range: 4.5 to 9.0), and 2.90% total salt (range: 1.55-7.40%). Autotrophic growth occurs via

methanogenesis using H<sub>2</sub> and CO<sub>2</sub>. No growth is observed when yeast extract, acetate,

methanol, or formate is used as the electron donor and carbon source, nor do they stimulate growth in the presence of H<sub>2</sub> and CO<sub>2</sub>. No growth is observed when nitrate (10 mM KNO<sub>3</sub>) or nitrogen gas (N<sub>2</sub>) are given as sole nitrogen sources.

The type strain JH146<sup>T</sup> (=DSM 27223<sup>T</sup>=KACC 18232<sup>T</sup>) was isolated from 26°C hydrothermal vent fluid from Axial Volcano at the depth of 1,520 m on the Juan de Fuca Ridge in the northeastern Pacific Ocean (45° 55' N, 129° 59' W). The G+C content of the type strain is 30.8% mol based on total genome calculations.

## **CHAPTER 5**

### **SUMMARY AND CONCLUSIONS**

This dissertation presented three research projects. The first examined the extent to which biological methane production on the subsurface of specific vent sites at Axial Volcano could be modelled based on methane production by laboratory cultures grown under hydrogen-limiting conditions. The second characterized the growth of a thiosulfate-, sulfur-, and nitrate-reducing thermophilic autotroph from Boardwalk vent at Endeavour to determine under what conditions it would be competitive for hydrogen with a thermophilic methanogen from the same site. The third examined the genome of a hyperthermophilic methanogen and whether its genome matched its observed phenotypic characteristics when grown in laboratory culture.

The first project focused on quantifying methane production by thermophilic and hyperthermophilic methanogens in both batch and continuous-flow culture in the laboratory. The conditions and variables for the laboratory experiments were determined by conducting microcosm experiments in the field using diffuse hydrothermal fluid. This allowed us to determine whether methanogens were capable of similar rates of growth and methane production without the supplementation found in laboratory media; whether their growth in environmental conditions was promoted by the addition of nitrogen; and whether the minimum hydrogen concentrations supported production of methane under environmental conditions. Having determined that the minimal laboratory media we used did not appear to be promoting methanogen growth at faster rates than those possible under environmental conditions, and that the major controls on their growth were

temperature and hydrogen concentration, we quantified their growth and methane production over a range of temperatures and hydrogen concentrations, as well as the interaction of temperature and hydrogen concentration. When we used the relationships between temperature and hydrogen concentration found in the lab to model methane production in the subsurface of Axial Volcano, we predicted that given the measured methane, hydrogen and methanogen concentrations at three target vent sites (Anemone, Marker 33, and Marker 113), these sites represent three different scenarios for methanogenesis at Axial Volcano. At the first, hydrogen concentrations were low and residence times short, permitting very few thermophilic methanogens to survive. At the second, hydrogen concentrations were high enough for significant methanogenesis and residence times long, so hyperthermophilic methanogens could use the hydrogen available. At the third, hydrogen concentrations were high and residence times relatively shorter, so methanogenesis was dominated by thermophilic and even moderately thermophilic methanogens. This model represented the first attempt to apply reactive transport modelling techniques to microbiological activity in ocean crust aquifer at hydrothermal vent sites.

The second project focused on determining minimum thiosulfate and hydrogen requirements for a hydrogenotrophic sulfur/thiosulfate-reducer isolated from diffuse hydrothermal vent fluid. Sulfur and sulfate reduction is more energetically favorable than methanogenesis under most vent conditions (McCollom and Shock, 1997; Houghton and Seyfried, 2010), and we wished to determine what concentrations of electron donors and acceptors would permit methanogens and sulfur/thiosulfate reducers to compete with each other. We grew our sulfur-reducing strain at different thiosulfate and hydrogen

concentrations, at its optimal temperature, on the same minimal medium as our methanogen experiments, and determined Monod kinetics and minimum requirements for both thiosulfate and hydrogen. We found that while our model sulfur-reducer could outcompete methanogens when electron acceptors (sulfur and/or thiosulfate) were not limiting, it would not outcompete them if they were. These two groups of organisms co-exist in the environment, probably due to this limitation.

The third project focused on looking at the newly-sequenced genome of a characterized but unnamed hyperthermophilic methanogen, *Methanocaldococcus* sp. JH146, from diffuse hydrothermal vent fluid at Axial Volcano. Methanogens have high levels of 16S rRNA gene sequence similarity and while this organisms had been characterized, it could not be determined to be a novel species on the basis of its 16S rRNA gene sequence. By doing whole-genome *in silico* comparisons, we could determine that its genome was significantly different from its closest relative and it was a new species, which we named *Methanocaldococcus bathoardescens*. *M. bathoardescens* also displayed different physiological characteristics from other *Methanocaldococci* and *Methanothermococci*, such as a positive response and lower methane production rate when grown with high nitrogen. Its major physiological difference from its closest relative (*Methanocaldococcus* sp. FS406-22) was that sp. FS406-22 fixes nitrogen and *M. bathoardescens* does not. We confirmed that *M. bathoardescens* lacks a number of genes in the nitrogen-fixation pathway and is not genetically capable of nitrogen fixation. However, we could not identify a genomic cause for its higher nitrogen requirements.

The main goal of this thesis has been to demonstrate how physiology allows us to better quantify habitability. There are many environments in which it is difficult or impossible

to directly measure rates for various microbiological processes that may be occurring, especially those which have a direct relationship with biogeochemical cycling – particularly the uptake of nitrogen, carbon, and sulfur into the biological sphere. Furthermore, there are environments beyond Earth of astrobiological interest where it may *never* be possible to make direct rate measurements. We are forced to categorize these environments on the basis of potential habitability using only remotely-sensed or limited data, in order to limit the number of search targets in our search for evidence of non-terrestrial life.

If we wish to make predictions about or understand these difficult to reach environments – on Earth or elsewhere – we can start by modelling the processes we think are occurring there and see whether our models match the outputs we observe for those environments, where those outputs are directly able to be sampled. But in order to model those processes, we must place constraints on the possible types of processes and rates that could be occurring. By looking at the physiological limits of organisms in the lab, under conditions that are as close as possible to those in the environment, we can provide constraints to our models. These relate directly to how organisms that we *know* currently exist in these environments are surviving. It is very important to remember that the overall rates of biogeochemical processes in these environments are ultimately constrained by chemistry and physics. We are very unlikely to find *Methanocaldococcus* in hydrothermal vents under the European ice, or in subsurface hydrothermal systems on Mars. But a hyperthermophilic, methane-producing organism in those environments will be operating under the same final constraints and probably behave in a lot of similar ways. By creating models which are based on the actual physiological rates of our target

organisms, and investigating how different organisms in these environments might compete for resources, as well as how molecular data and genomes can inform us about their capabilities, we not only enable ourselves to better understand environments on Earth – we can draw up guidelines for the search for life elsewhere.



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